Organ-specific Expression of Insulin-like Growth Factor I (IGF-I) During Early Development of the Bony Fish *Oreochromis niloticus*, the Tilapia

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Foreword

This Ph.D. thesis is based on the results published in international, peer-reviewed, scientific journals. It is presented in three chapters: the first chapter gives an introduction to the entire work and the following two chapters correspond to the published papers.

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Summary

Insulin-like growth factor I (IGF-I) plays a key role in the complex system that regulates vertebrate growth, differentiation and reproduction. The major source of endocrine IGF-I is the liver, which synthesizes and secretes IGF-I after stimulation by growth hormone (GH) from the anterior pituitary. Circulating IGF-I binds to specific receptors at peripheral sites and exert its physiological effects. In mammals, IGF-I is also synthesised in various organs and has the ability to act in a paracrine or an autocrine manner in addition to the endocrine route.

In order to get a first impression of the potential local actions of IGF-I during ontogeny, it is important to know the IGF-I synthesis sites. The present study approaches this problem by investigating the dynamics of IGF-I expression during early development of the bony fish *Oreochromis niloticus*, the Nile tilapia. *O. niloticus* is a major commercial tropical fish, cultured in increasing numbers throughout the world and, therefore, any insight in the regulation of its ontogeny is of special importance. The studies were carried out with monosex progenies of *O. niloticus*, in the early stages of development starting from the day of hatching, i.e. at 4 days post fertilization (DPF), until the adult life. Specific antisera raised against human IGF-I and a fluorescent detection system were used to investigate IGF-I peptide localisation. IGF-I mRNA was detected by digoxigenin labelled RNA probes complementary to tilapia IGF-I mRNA sequence and nitroblue tetrazolium colour reaction. Crossreactivity and specificity of binding were verified for both antisera and RNA probes.

In liver, IGF-I mRNA was expressed already at the day of hatching, 4 DPF, indicating the physiological impact of circulating IGF-I during development.

IGF-I also appeared very early in tissues which are highly involved in growth, such as cartilage and skeletal muscle. At 4 DPF, IGF-I mRNA and peptide were already present in chondrocytes of cartilage of various locations, with increasing intensity towards the growing regions, and also found in adults. The onset of IGF-I production in skeletal muscle was also at 4 DPF. It showed a maximum around 10-29 DPF, decreased later but persisted throughout life.

In the gastro-intestinal tract it was expressed at 5 DPF in the intestine and at 9 DPF in the stomach. During the first weeks of development, the IGF-I-immunoreactive material was mainly located in the apex of epithelial cells, possibly suggesting its release into the lumen.

Around 17 DPF, additional IGF-I containing mucosal cells appeared which showed the typical appearance of endocrine cells. These persisted throughout life. In the exocrine pancreas, the expression of IGF-I started at 4 DPF and occurred until 90 DPF. IGF-I was found in non-insulin cells of endocrine pancreas from 6 DPF as well as during adult life.

In the gills, IGF-I mRNA appeared in chloride cells at 6 DPF. The early appearance of IGF-I may suggest that it exerts functions, such as to influence growth and maintenance of the filament epithelium, in addition to its impact in the regulation of plasma osmolality and gill Na^+, K^+-ATPase activity as described in adult fish. In the kidney, that is also involved in osmoregulation, IGF-I production started at 8 DPF in epithelial cells of renal tubules and ducts and persisted until adult age.

In the heart, IGF-I mRNA and peptide appeared at 13 DPF in cardiomyocytes. The expression of IGF-I was detectable until 70 DPF but absent from later stages and adults indicating a particular physiological impact of IGF-I during heart development and growth.

IGF-I appeared in epidermal cells at 5 DPF. The number of IGF-I producing cells first increased with age reaching the highest level around 19-29 DPF. Later some superficial and basal epidermal cells exhibited IGF-I mRNA likely suggesting that IGF-I in skin is not only involved in proliferation but also in repair mechanisms and wound healing.

IGF-I mRNA was expressed in virtually all neurons of brain and ganglia from 6 to 29 DPF, their number decreasing with age. IGF-I immunoreactivity was detected in neurohypophysis at 17 DPF and in adenohypophysis at 40 DPF, while the mRNA appeared only in adenohypophysis at 28 DPF. Thus, IGF-I in the neuropituitary likely originates from neuronal perikarya within the hypothalamus which exhibited IGF-I-immunoreactivity.

In the male and female gonad anlagen, IGF-I mRNA was detectable in somatic cells already after its first appearance around 7 DPF, followed by the peptide at 9-10 PDF. In undifferentiated primordial germ cells IGF-I peptide, but not mRNA, was detected at 7 DPF, suggesting that it may be derived from somatic cells or be of maternal origin. IGF-I mRNA appeared at 29 DPF in female and around 51-33 DPF in male germ cells, which coincides with the onset of meioses in tilapia ovaries and testis. In adult testis, IGF-I mRNA and peptide occurred in spermatogonia and spermatocytes and in Leydig cells, the latter indicating a role of IGF-I in the synthesis of male sex steroids. In adult ovary, IGF-I mRNA and IGF-I peptide

were present in small and previtellogenic oocytes and occurred in granulosa and theca cells of later follicles.

Thus, while liver synthesizes IGF-I throughout life, in parenchymal cells of most organs investigated, such as epithelial cells of the gastro-intestinal tract, acinar cells of the exocrine pancreas, skeletal muscle cells, cardiomyocytes, renal tubular and duct cells, neurones of the central and peripheral nervous system, and skin cells the expression of IGF-I was more pronounced during ontogeny than in juvenile and adult life. These results suggest a high functional impact of local IGF-I in early fish organogenesis, growth and metabolism by auto/paracrine means of regulation. The findings, thus, support the idea of a dual mode of action for IGF-I: as endocrine hormone and as local para/autocrine hormone.

Zusammenfassung

Insulin-like growth factor I (IGF-I) spielt eine Schlüsselrolle bei der komplexen Regulation des Wachstums, der Differenzierung und der Vermehrung der Wirbeltiere. Die Hauptquelle des endokrinen IGF-I ist die Leber. Nach Stimulation durch Wachstumshormon aus dem Hypophysenvorderlappen synthetisiert und sezerniert die Leber IGF-I. Das zirkulierende IGF-I bindet in der Peripherie an spezifische Rezeptoren und ruft so seine physiologischen Wirkungen hervor. Bei Säugern wird IGF-I darüber hinaus auch in zahlreichen anderen Organen gebildet und kann zusätzlich zu seiner endokrinen Wirkung auch parakrine und autokrine Wirkungen hervorrufen.

Um eine Vorstellung von den möglichen lokalen IGF-I-Wirkungen während der Ontogenese zu erhalten, ist es wichtig, die Syntheseorte von IGF-I zu kennen. In der vorliegenden Studie wird daher die Dynamik der IGF-I-Expression während der frühen Entwicklung des Knochenfisches *Oreochromis niloticus*, der Nil-Tilapia, untersucht. *O. niloticu*s ist ein tropischer Fisch von wirtschaftlicher Bedeutung, der weltweit in zunehmenden Mengen gezüchtet wird. Kenntnisse über die Regulation der Ontogenese sind daher von speziellem Interesse. Die Studien wurden an Nachkommen gleichen Geschlechts vom Tag des Schlüpfens, i.e. vom 4. Tag nach der Fertilisierung (DPF), bis zum Erwachsenenalter durchgeführt. Für die Gewebslokalisation von IGF-I Peptid wurde eine Fluoreszenzmethode mit spezifischen Antiseren gegen menschliches IGF-I benutzt. IGF-I mRNA wurde mit Digoxigenin-markierten RNA Proben von komplementärer Sequenz zur Tilapia IGF-I mRNA und durch eine Farbreaktion mit Nitroblau-Tetrazolium nachgewiesen. Sowohl für Antiseren als auch für RNA Proben wurden Kreuzreaktivität und Spezifität getestet.

In der Leber wurde IGF-I mRNA bereits am Tag des Schlüpfens, 4 Tage nach der Fertilisation, exprimiert, ein Hinweis darauf, dass zirkulierendes IGF-I bereits während der frühen Entwicklung von Bedeutung ist.

IGF-I erschien auch schon früh im Knorpel und im Skelettmuskel. So waren IGF-I Peptid und IGF-I mRNA bereits 4 Tage nach Fertilisation in Chondrozyten verschiedener Knorpelgewebe nachweisbar, und zwar mit zunehmender Intensität zu den Wachstumszonen des Knorpels hin. Auch im Skelettmuskel begann die IGF-I Produktion am 4. Tag nach der

Fertilisation. Das Maximum lag zwischen dem 10. und 29. Tag nach Fertilisation. Später fiel die IGF-I Produktion ab, hielt jedoch während der gesamten Lebensspanne an.

Innerhalb des Magen-Darm-Trakts wurde IGF-I im Darm am 5., im Magen am 9.Tag nach der Fertilisation exprimiert. Während der ersten Wochen der Entwicklung war immunreaktives IGF-I vorwiegend an der apikalen Seite der Epithelzellen lokalisiert, möglicherweise ein Hinweis auf seine Freisetzung ins Lumen. Um den 17. Tag nach Fertilisation erschienen zusätzlich IGF-I enthaltende Mucosazellen, die das typische Aussehen endokriner Zellen zeigten. Diese Zellen blieben während der gesamten Lebensspanne bestehen. Im exokrinen Pankreas war die IGF-I-Expression vom 4. bis zum 90. Tag nach Fertilisation nachweisbar. IGF-I wurde ab dem 6. Tag nach Fertilisation in nicht Insulin-produzierenden Zellen des endokrinen Pankreas gefunden, ebenso im Erwachsenenalter.

In den Kiemen erschien IGF-I mRNA am 6. Tag nach Fertilisation in den Chloridzellen. Dieses frühe Auftreten könnte darauf hinweisen, dass IGF-I nicht nur für die Regulation der Plasmaosmolalität und die Aktivität der Na^+, K^+ -ATPase der Kiemen, wie bei erwachsenen Fischen beschrieben, nötig ist, sondern auch für das Wachstum und die Aufrechterhaltung des Filamentepithels. In der Niere, die ebenfalls an der Osmoregulation beteiligt ist, begann die IGF-I Produktion 8 Tage nach der Fertilisation in Epithelzellen der Nierentubuli und Sammelrohre und hielt bis zum Erwachsenenalter an.

Im Herz erschienen IGF-I mRNA und Peptid 13 Tage nach Fertilisation in den Kardiomyozyten. Die Expression bestand bis zum 70. Tag nach Fertilisation, fehlte jedoch später und im Erwachsenenalter. Möglicherweise weist dies auf eine spezielle physiologische Rolle während Herzentwicklung und Herzwachstums hin.

In Epidermiszellen trat IGF-I am 5. Tag nach der Fertilisation auf. Zunächst nahm die Zahl der IGF-I- immunreaktiven Zellen mit dem Alter zu und erreichte ein Maximum zwischen dem 19. und 29. Tag nach der Fertilisation. Später enthielten einige oberflächliche und basale epidermale Zellen IGF-I mRNA, wahrscheinlich ein Hinweis darauf, dass IGF-I in der Haut nicht nur die Zellproliferation stimuliert, sondern auch an Reparaturmechanismen und an der Wundheilung beteiligt ist.

IGF-I mRNA wurde vom 6. bis zum 29. Tag nach Fertilisation in praktisch allen Neuronen des Gehirns und der Ganglien exprimiert, wobei die Zahl der IGF-I exprimierenden

Zellen mit dem Alter abnahm. In der Neurohypophyse war immunoreaktives IGF-I am 17., in der Adenohypophyse am 40. Tag nach Fertilisation nachweisbar, während die IGF-I mRNA nur in der Adenohypophyse am 28. Tag nach Fertilisation erschien. Daher stammt das IGF-I in der Neurohypophyse wahrscheinlich aus neuronalen Perikaryen im Hypothalamus, die immunreaktives IGF-I aufwiesen.

In der männlichen und weiblichen Gonadenanlage war IGF-I mRNA in den somatischen Zellen bereits kurz nach deren Erscheinen um den 7. Tag nach Fertilisation nachweisbar, IGF-I Peptid an den Tagen 9-10. In undifferenzierten primordialen Keimzellen war IGF-I Peptid, nicht aber IGF-I mRNA, am Tag 7 nach Fertilisation nachzuweisen, ein Hinweis darauf, dass es aus somatischen Zellen stammt oder mütterlichen Ursprungs ist. IGF-I mRNA erschien am 29. Tag nach Fertilisation in weiblichen und zwischen dem 51. und 53. Tag in männlichen Keimzellen, und damit gleichzeitig mit dem Beginn der Meiose in den Ovarien und Hoden. In den Hoden erwachsener Tiere fanden sich IGF-I mRNA und IGF-I Peptid in Spermatogonien, Spermatozyten und Leydig'schen Zwischenzellen. Letzterer Befund dürfte auf eine Rolle von IGF-I bei der Synthese männlicher Sexsteroide hindeuten. Im Ovar erwachsener Tiere lagen IGF-I mRNA und IGF-I Peptid in kleinen und prävitellogenen Oozyten vor, in späteren Follikelstadien in Granulosa- und Thekazellen.

Zusammenfassend lässt sich folgendes festhalten: Während die Leber über die gesamte Lebensspanne IGF-I synthetisiert, war die Expression von IGF-I in den Parenchymzellen der meisten untersuchten Organe - so in den Epithelzellen des Magen-Darm-Trakts, in den Azinuszellen des exokrinen Pankreas, in den Skelettmuskelzellen und den Kardiomyozyten, in den Nierentubulus- und Sammelrohrzellen, in den Neuronen des zentralen und peripheren Nervensystems und in Hautzellen - während der Ontogenese stärker ausgeprägt als im juvenilen und Erwachsenenzustand. Diese Befunde sprechen für eine zweifache Wirkungsweise von IGF-I: die eines endokrinen und die eines auto/parakrinen Hormons.

General Introduction

IGF-I system

Overview

Insulin-like growth factor-I (IGF-I) is a member of the insulin/IGF-I family of hormones that play an important role in metabolism, growth and development. The insulin/IGF-I family is comprised of ligands (insulin, IGF-I and IGF-II), six well characterized binding proteins (IGFBP-1 through -6), and cell surface receptors that mediate the actions of the ligands (insulin receptor, IGF-I receptor and the IGF-II receptor, that is the mannose-6-phosphate [M6P] receptor with additional IGF-II binding site) (Fig. 1).

The IGFs were originally identified in 1957 by their ability to stimulate ^{35}S incorporation into rat cartilage and termed "sulfation factor" (Salmon and Daughaday, 1957). Experiments *in vivo* as well as *in vitro* demonstrated the existence of an intermediate component responsible for the incorporation of ^{35}S in the epiphyseal cartilage after the stimulation by growth hormone (GH) (Denko and Bergenstal, 1955; Murphy et al., 1956; Daughaday and Reeder, 1966). Subsequent studies expanded the spectrum of biological actions of the sulfation factors, including insulin-like activity that could not be neutralized by antisera against insulin (Froesch et al., 1963), and mitogenic effects on HeLa cells (Salmon and Hosse, 1971) and embryo fibroblasts (Dulak and Temin, 1973b; Dulak and Temin, 1973a). In the light of these and other findings the new term "somatomedin" was proposed in 1972 (Daughaday et al., 1972), denoting that this compounds mediated effects of GH.

Purification yielded a GH-responsive fraction, somatomedin C and non-responsive somatomedin A. In 1978, the active substances were isolated and sequenced from human serum (Rinderknecht and Humbel, 1976; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). They were named insulin-like growth factors (IGFs) on the basis of their functional (glucose uptake by fat cells and muscles) and sequence (approximately 50% amino

acid sequence) similarity to insulin. As it became clear that the somatomedins were a part of a larger superfamily of proteins, the more appropriate term IGF was adopted (Daughaday et al., 1987).

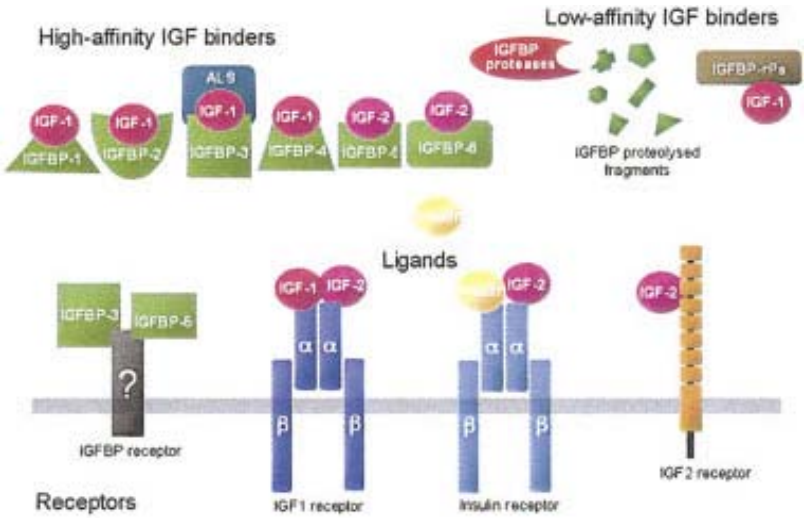

Fig. 1 The IGF family and related signaling molecules (Dupont and Holzenberger, 2003).

Liver is the main source of circulating IGF-I. Under the regulation of GH IGF-I is synthesized and secreted from the liver into the circulation and acts on numerous peripheral tissues and cell types, exerting metabolic and mitogenic actions. This is known as the somatomedin hypothesis, which states that growth-promoting function of GH is mediated by IGF-I. Circulating IGF-I also provides a negative feedback on GH secretion from the anterior pituitary gland (Berelowitz et al., 1981).

In addition to its endocrine mechanism of action, IGF-I also acts in local autocrine or paracrine manners. This mode of IGF-I action is apparent from observations that IGF-I is produced in numerous extrahepatic sites (D'Ercole et al., 1980; Roberts et al., 1987; Han et al., 1988), and by the fact that even a 75% reduction of circulating IGF-I has little effect on postnatal growth in mice (Sjogren et al., 1999; Yakar et al., 1999). Although locally produced IGF-I may be regulated by other factors as well, GH was shown to affect its production in several organs (Lowe et al., 1987; Lowe et al., 1988). It is not yet entirely clear what is the importance of local versus circulating IGF-I, and what are the exact mechanisms of regulation of IGF-I action.

IGF-I

General

Structurally, the IGFs belong to the insulin superfamily of polypeptides which also includes insulin, relaxins, and a number of insulin-like proteins (INSL) identified in vertebrates and invertebrates, including, 7 drosophila insulin-like peptides, DILP (Brogiolo et al., 2001); and 37 insulin-like ligands, INS in *Cenorhabditis elegance* (Pierce et al., 2001). They exhibit a broad functional diversity and a high degree of structural conservation (Fig. 2)

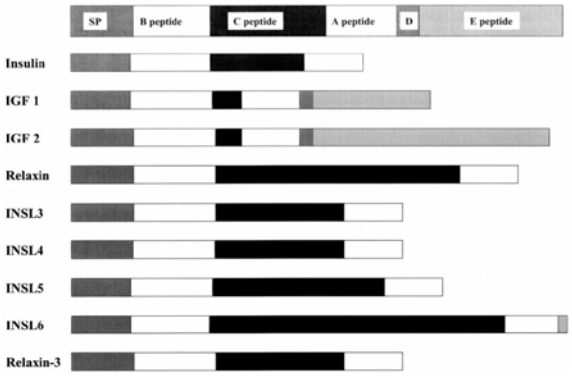

Fig. 2 Schematic representation of gene products of the insulin family in vertebrates. The peptide domains are presented in relative scale (Lu et al., 2005).

Mammalian IGF-I is a 70 amino acid peptide, with a molecular mass of about 7.5 kDa, structurally related to proinsulin (Fig. 3). IGF-I shares with insulin the A and B domains that are linked by two disulphide bonds. Like proinsulin, the A and B domains of the IGFs are connected through a C domain, but the C domain of IGF-I is considerably shorter than the C peptide of proinsulin and exhibits a different sequence. Furthermore, in contrast to insulin, the C domains of the IGFs are not proteolytically removed during prohormone processing and, therefore, the mature IGF molecules are single-chain polypeptides. In further contrast to insulin, the mature IGFs contain additional six to eight-residue carboxy-terminal D domains. Finally, the prohormones of the IGFs show further carboxy-terminal extensions, the E peptides, which are gradually cleaved during translational processing (Reinecke and Collet, 1998).

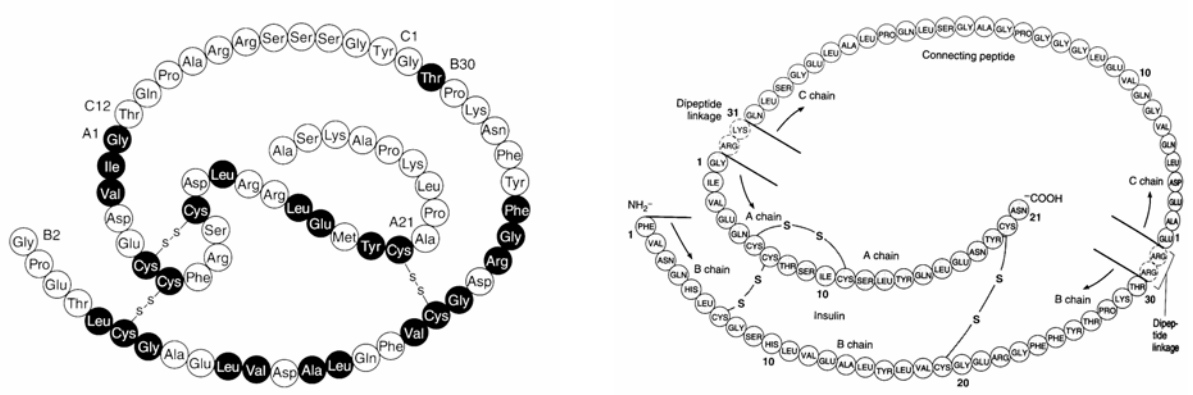

Fig. 3 Amino acid sequence of human IGF-I (left) and proinsulin (right). Shared amino acids are highlighted in black (Hadley, 1996; Greenspan and Strewler, 1997).

Crystallographic studies revealed the structure of IGF-I as similar to insulin, but also substantial dissimilarities resulting from of C chain-derived C-loop on IGF-I were found (Brzozowski et al., 2002) (Fig. 4).

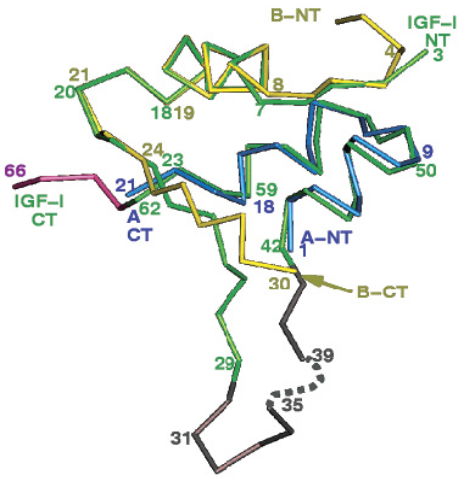

Fig. 4 Superimposed 3-dimensional structure of the human IGF-I and insulin molecules. Insulin is represented by blue - A chain and yellow - B chain. IGF-I is represented mostly by green, with brown - C domain and purple - D domain (Brzozowski et al., 2002).

Fish

Strong evidence for the presence of IGF-like activity in fish serum was demonstrated already in the late seventies (Shapiro and Pimstone, 1977; Komourdjian and Idler, 1978). The first fish IGF-I sequence was established for Coho salmon (Cao et al., 1989). Since then, full length as well as partial sequences of IGF-I have been deduced in many fish species (comprehensive list in: (Wood et al., 2005)), including the tilapia species: *Oreochromis mosambicus* (Reinecke et al., 1997) and *Oreochromis niloticus* (Cruz et al., 2006). Sequence comparison for various classes of vertebrates revealed that the IGF-I sequence is highly conserved throughout evolution. Mature teleost IGF-I consists of 68-70 amino acids depending on the species and shows an overall 72-81 % sequence similarity with human IGF-I. Not only sequence, but also biological actions of IGF-I have been shown to be highly conserved between the different classes of vertebrates. Numerous studies indicate a high potency of human IGF-I to induce ^{35}S incorporation in cartilage of various teleost species. Similarly, activities of fish and mammalian IGF-I are almost equally potent in tissues of mammals (Duan, 1998).

The structure of the fish IGF-I genes tends to be much more compact and simple as compared to mammals. In contrast to the human IGF-I gene, which contains six exons and spans 100 kilobases (kb), the single fish IGF-I gene is composed of five exons and spans around 20 kb (Wood et al., 2005). Multiple mRNA transcripts have been identified in numerous teleost species including tilapia (Reinecke et al., 1997).

IGF-I Receptor

General

The receptor for IGF-I (IGF-1R) represents a tyrosine kinase-linked transmembrane receptor. The IGF-1R is synthesized as a single peptide of 1367-amino acid sequence and proteolytically cleaved into α and β subunits linked by disulfide bonds. Two α chains are joined by secondary disulfide bonds to form the mature $(\alpha\beta)_2$−receptor. The α subunits are extracellular and contain a cysteine-rich domain. The β subunit has a short extracellular

domain, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain and ATP-binding site (Fig. 5).

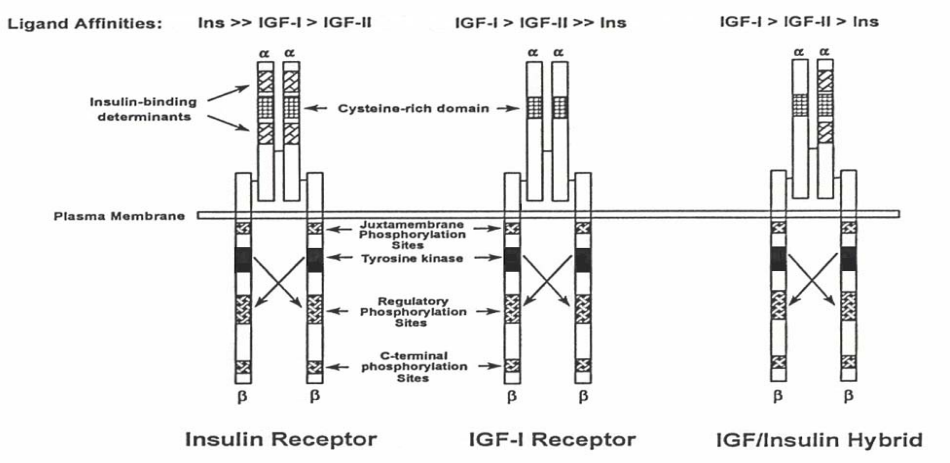

Fig. 5 Schematic representation of receptors for insulin, IGF-I and IGF-I/insulin hybrid receptors (Jones and Clemmons, 1995).

Despite its name the IGF-1R binds both IGF-I and IGF-II with high affinity, and both IGF peptides appear capable of activating tyrosine kinase by binding to the receptor. Insulin can also bind to the IGF-1R but with 100- to 1000-fold less affinity, which explains the relatively weak mitogenic effects of insulin (Williams and Larsen, 2002). In addition, the IGF receptor $\alpha\beta$-pair can form heterodimers with the insulin receptor $\alpha\beta$-pair, IGF-1R (Fig. 5). These hybrids only bind IGF-I, which points to the importance of cysteine rich regions as necessary for IGF-I binding.

Upon activation by ligand binding, tyrosine kinase of one β-subunit phosphorylates residues on the reciprocal β-subunit. Autophosphorylation of the receptor results in recruitment of multiple adaptor and docking proteins, such as the members of insulin receptor substrate family (IRS-1 through IRS-4). Recruitment of IRS and other substrates initiates a cascade of further phosphorylation events, involving multiple second-messengers. For example, recruitment of SH2 domain-containing proteins can activate the monomeric G protein Ras, triggering the activation of the mitogen-activated protein kinase (MAPK) signaling pathway. The alternative pathway involves the activation of phosphatidylinositol-3 kinase (PI3-kinase) or protein kinase B (PKB/Akt) signal transduction. The different

pathways ultimately influence nuclear transcription and gene expression. These, depending on the cell type and cellular context involved, can lead to mitogenic activity, cell proliferation, prevention of apoptosis and glucose uptake (Fig. 6) (Wood et al., 2005).

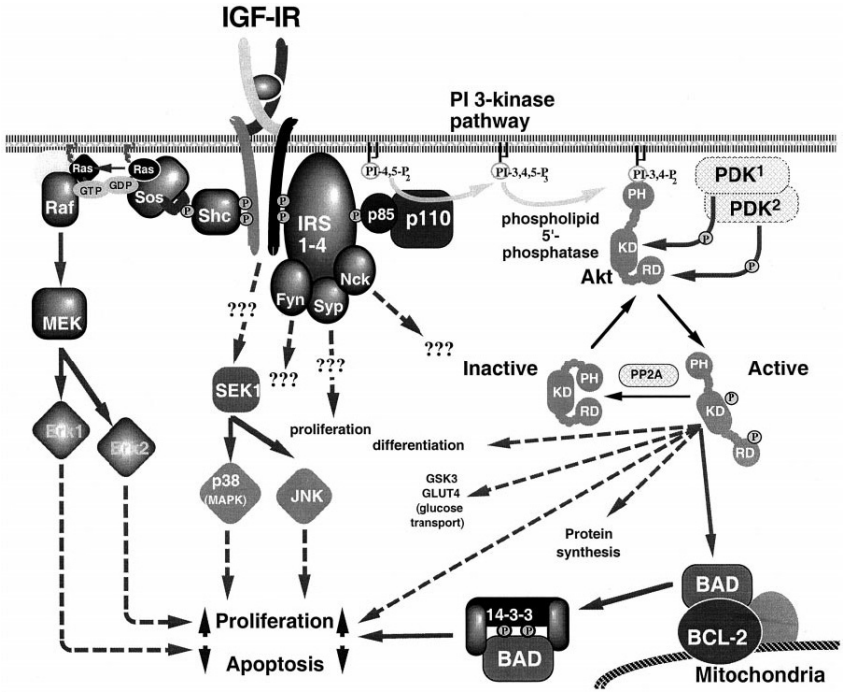

Fig. 6 Signal transduction cascades emanating from activation of the IGF-I receptor (Le Roith et al., 2001)

Fish

The primary structure of the IGF-1R is known for a number of fish species. There is a great deal of sequence similarity between mammalian and fish IGF-1R, with the cysteine rich region being the most conserved. Two types of IGF-1R (IGF-1Ra and IGF-1Rb) have been described in rainbow trout (Greene and Chen, 1999a), zebrafish (Maures et al., 2002) and Japanese flounder (Nakao et al., 2002). Some differences in the expression pattern of the IGF-1R subtypes have been observed. Knockdown studies showed large degrees of functional overlap and additive effects, but also some specific functions of subtypes during the embryonic development in zebrafish (Schlueter et al., 2006).

Binding studies of mammalian and fish IGF-I demonstrated a high affinity of the fish IGF-1R to both ligands and, therefore, functional similarity to the mammalian IGF-1R.

IGF-Binding Proteins

General

The majority of the IGFs present in extracellular fluids are found in a complex with specific, high affinity insulin-like growth factor-binding proteins (IGFBPs). There are six distinct IGFBPs characterized in mammals, 24-45 kDa proteins, which share around 50 % sequence similarity to each other (Rechler, 1993). Structurally all IGFBPs share conserved amino N- and carboxy C- terminal domains, and a variable central L-domain (Fig. 7).

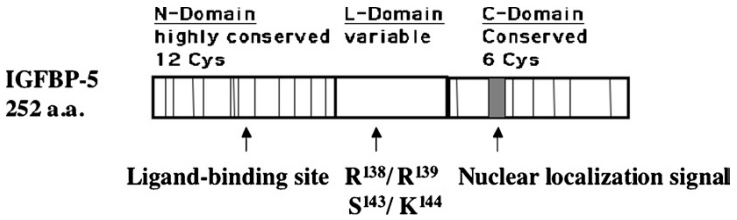

Fig. 7 Structure of IGFBP (IGFBP5) (Duan and Xu, 2005).

The N-domain serves as binding site for the IGFs and has 12 conserved cysteine residues. The C-domain has the capability of binding other molecules, such as ASL for IGFBP-3 and IGFBP-5. The central, linker domain represents the site for post-translational modification (Duan and Xu, 2005).

The functions of the IGFBPs can be divided in three categories. They serve as carriers for the IGFs and modulators of IGF action and seem to exert IGF-independent bioactivity (Williams and Larsen, 2002).

Carrier: In the normal adult serum 75-80 % of all IGF peptides circulate in a 150 kDa ternary complex consisting of one molecule of IGF, one molecule of IGFBP-3 and one molecule of 88 kDa protein termed the acid-labile subunit (ASL) (Baxter and Martin, 1989; Leong et al., 1992). A ternary structure with IGFBP-5 instead of IGFBP-3 is also present, however, at much less extent (Twigg et al., 1998; Baxter et al., 2002). Because the large molecular mass IGF complex cannot cross the vascular endothelial barrier and is protected from proteolytic degradation it acts as a reservoir of IGFs in the serum. Thereby it extends half-life of IGF peptide from approximately 10 minutes for IGF alone to 1-2 hours in the IGF-

IGFBP-3 binary complex to 12-15 hours for IGF in the ternary complex (Guler et al., 1989; Zapf, 1995).

Modulator: The binding affinity of the IGF peptides to the IGFBPs is higher than that to the IGF receptors, which implies that the IGFBPs can modulate IGF-binding to its receptors, thereby modulating IGF biological action (Oh et al., 1993; Jones and Clemmons, 1995; Firth and Baxter, 2002). IGFBPs have been shown to inhibit various actions of IGF-I on cell cultures (Okajima et al., 1993; Williams and Larsen, 2002). IGFBP proteases have been identified and postulated to play a role in altering IGF availability by lowering the affinities of the IGFBPs for their ligand, thereby mobilizing IGFs from the circulating reservoir and facilitating the precise delivery of the ligand to the cell membrane receptors (Lamson et al., 1993; Cohen et al., 1994; Lee and Rechler, 1995; Lee and Rechler, 1996). IGFBPs also are thought to prevent the insulin-like activity of the IGFs (Mohan and Baylink, 2002).

Independent: Intriguingly, an additional level of complexity in IGFBP function seems to emerge: IGF-independent biological actions have now been documented for selected members of the IGFBP family. That includes effects on cell migration, cell growth and apoptosis. Elucidating the IGF-independent functions of IGFBPs is currently the focus of considerable research interest (Duan, 2002; Kelley et al., 2002; Lee and Cohen, 2002; Mohan and Baylink, 2002).

Fish

One or more types of IGFBPs have been identified in blood and tissues of several fish species (Kelley, 2006). Binding capability to IGF-I and IGF-II but not to insulin has been demonstrated for some of them (Duan et al., 1999; Bauchat et al., 2001; Shimizu et al., 2003). The most abundant type of fish IGFBP in serum of tested species is an around 41 kDa molecule, which is regulated by GH and considered as the likely homolog to mammalian IGFBP-3 (Shimizu et al., 2003). Despite considerable advances in recent years, data regarding the function and functional and structural relationship of fish IGFBPs to their mammalian orthologs is not conclusive to date (Reinecke et al., 2005).

IGF Function in Fish

Growth

The original phenomenon which demonstrated the growth-promoting function of IGF-I was the ability to stimulate ^{35}S incorporation in growing cartilage. This ability of the IGFs was demonstrated in numerous teleost species (Wood et al., 2005). Besides sulphation, IGF-I has been implicated in many growth-related processes in fish. It stimulates DNA synthesis as demonstrated by [^{3}H]thymidine incorporation into cartilage (Duan and Hirano, 1992), gonads (Loir and Le Gac, 1994; Srivastava and Vanderkraak, 1994), muscles (Castillo et al., 2004) and embryonic cells (Pozios et al., 2001). IGF-I stimulates protein synthesis (Negatu and Meier, 1995; Degger et al., 2000; Gallardo et al., 2001). Mitogenic effects of IGF-I were shown by the induction of proliferation of retinal cells in cichlids and goldfish (Mack and Fernald, 1993; Otteson et al., 2002). Somatic growth was reported after IGF-I infusion in salmonids (McCormick et al., 1992).

Additional data for the role of IGF-I and the GH−IGF-I axis in teleost growth have been obtained from studies of stunted fish, which show retarded growth after abrupt fresh- to saltwater transfer (Clarke et al., 1977). GH resistance and IGF-I deficiency have been demonstrated in stunted fish (Bolton et al., 1987; Gray et al., 1992; Duan et al., 1995), linking GH−IGF-I axis with the regulation of growth in fish. Stunted fish are thought to suffer from malnutrition caused by impaired nutrient uptake from food (Collie and Stevens, 1985). Indeed, there is also a correlation between nutritional status, GH treatment, IGF-I levels and body size or growth rate in fish (Reinecke, 2006). As in other vertebrates, in teleost fish starvation causes increase in circulating GH, but reduced GH sensitivity in liver (Gray et al., 1992; Small et al., 2006). This phenomenon is associated with decreased IGF-I levels in fish serum (Moriyama et al., 1994; Small and Peterson, 2005). Conversely, increased food rations or high temperature and the resulting increased growth rate correlate with elevated IGF-I and decreased GH levels in blood (Perez-Sanchez et al., 1995; Cruz et al., 2006).

Development

In 1992, Duguay and colleagues showed IGF-I mRNA transcripts in whole embryo of salmonids, six weeks post fertilisation, indicating that IGF-I is present during fish

embryogenesis and development (Duguay et al., 1992). Since then, IGF-I mRNA and peptide, as well as IGF-I receptor have been detected in embryos and larvae of various stages in a number of fish species, indicating a functional role for IGF-I in fish development (Reinecke, 2006).

Recent IGF-1R knockdown studies in fish confirm the importance of the IGF-signaling system during embryonic development. Inactivation of either of two types of IGF-1R results in delayed development for zebrafish embryo, abnormal morphogenesis and dysfunction of several organs, and it is 100 % lethal after 96 hours post fertilization (Schlueter et al., 2006). The knockdown of zebrafish IGF-1R alters the expression pattern of genes responsible for embryonic development of central nervous system and organization of body plan, which causes reduced body size and loss of anterior structures (Eivers et al., 2004).

Osmoregulation

Osmoregulation represents a considerable challenge for euryhaline or diadromous fishes, adapted to strong variation of water salinity (McCormick, 2001). They need to modify or even reverse salt balance depending on the environment. Several hormones are thought to direct these osmoregulatory processes in fish. While prolactin is considered as the "freshwater hormone" that induces freshwater adaptability, cortisol is important during the seawater acclimation (Utida et al., 1972). There are strong data that suggest the involvement of the GH−IGF-I axis in induction of seawater tolerance in fish (reviewed in: McCormick, 2001; Sakamoto and McCormick, 2006). Seawater challenge elevates GH and IGF-I levels (Lindahl et al., 1985; Sakamoto and Hirano, 1993; Yada et al., 1994), whereas administration of IGF-I enhances the ability to maintain plasma osmolality (Clarke et al., 1977; McCormick et al., 1991; Madsen et al., 1995; McCormick, 1996) and improves the tolerance to water salinity (Mancera and McCormick, 1998). The mechanisms responsible for these osmoregulatory activities involve the stimulation of Na^+,K^+-ATPase activity in chloride cells and the increase of their numbers in gills (Madsen and Bern, 1993; McCormick et al., 1995; Xu et al., 1997; Seidelin et al., 1999), which improves the salt-secretory capacity of gills and facilitates water retention from urinary bladder, gut and kidney (Utida et al., 1972). Changes in GH and IGF-I plasma levels, along with increase of prolactin levels, have also been observed after freshwater transfer in tilapia (Riley et al., 2003).

IGF-I along with GH, insulin and thyroxine have been all implicated in parr-smolt transformation in juvenile salmonids. This process represents metamorphic modifications associated with freshwater to salt water transfer during migration of anadromous fish species to the sea. During this process elevated IGF-I levels were found in liver (Duan et al., 1995), additionally in gills (Duguay et al., 1994), and in gills and kidney (Sakamoto and Hirano, 1993), but not in brain, muscle or ovary (Duguay et al., 1994; Duan et al., 1995).

Reproduction

The IGF system is strongly involved in reproductional processes in fish. The IGFs and the IGF-1R have been found in testes and ovaries of many teleost species. In females peptides have been localised in the cytoplasm of young oocytes, granulosa cells and steroid-producing theca cells of fish ovary (Schmid et al., 1999; Perrot et al., 2000). In the testes, IGF-I has been found in Sertoli cells, spermatocytes and Leydig cells (LeGac et al., 1996; Reinecke et al., 1997; Perrot et al., 1999). In the ovary IGF-I stimulates oocyte maturation (Kagawa et al., 1994; Negatu et al., 1998) and thickening of gap junctions in the granulosa layer (Patino and Kagawa, 1999). In males, IGF-I stimulates spermatogenesis (Loir and Le Gac, 1994; Loir, 1999) and enhances spermatogenic action of testosterone (Nader et al., 1999).

Another aspect of IGF-I action with respect to reproduction is its ability to influence the secretion of reproductive hormones. It has been shown that IGF-I inhibits testosterone production, whereas it stimulates the synthesis of estradiol, progesterone (Maestro et al., 1997) and maturation-inducing steroid (MIS) (Weber and Sullivan, 2000). IGF-I also stimulates the release of gonadotropins from the pituitary, such as luteinizing hormone (LH) (Huang et al., 1998), and enhances pituitary response to the activities of hypothalamic gonadotropin releasing hormones (Weil et al., 1999; Baker et al., 2000).

Tilapia model system

The Nile tilapia, *Oreochromis niloticus* (Fig. 8) belongs to the tentative Tribe Tilapiini, represented by the three main genera, *Tilapia*, *Sarotherodon* and *Oreochromis* (Trewavas, 1983). Tilapiini along with Haplochromini represent two major groups in the Family Cichlidae (Regan, 1920). The Family Cichlidae is classified in the Order Perciformes, the largest order of vertebrates, with more then 9200 species (Moyle and Cech, 2004).

Fig. 8 Nile tilapia, *Oreochromis niloticus* 90 DPF.

The Nile tilapia is a tropical, freshwater fish which occupies a wide variety of freshwater habitats such as rivers, lakes, sewage canals and irrigation channels (Bailey, 1994). It is also found in the brackish water of river deltas and estuaries. Tilapia is omnivorous and feeds on phytoplankton, aquatic plants, small invertebrates, benthic fauna, detritus, etc.

It is naturally distributed in Africa: coastal rivers of Israel, Nile from below Albert Nile to the delta, Jebel Marra, Lake Chad basin and the rivers Niger, Benue, Volta, Gambia and Senegal [www.fishbase.org]. The Nile tilapia has been introduced worldwide for aquaculture and as a result, can be found in various sites outside its area of natural

distribution, not only in Africa, but all around the world. Several countries report adverse ecological impact after introduction.

The tilapia is an important commercial fish. It has been cultured already in ancient Egypt, as indicated by its frequent occurrence in ancient Egyptian art, dating back for over 4000 years. From the middle of 20th century, tilapia aquaculture started spreading worldwide and currently is performed in more than 75 countries in Africa, Asia and the Americas, with China as the biggest producer. Thus, the tilapia is a substantial aquaculture fish, reaching worldwide production of 1.5 million tones in 2004 (FAO, 2005), which makes it one of the most cultured fish along with carp and salmon.

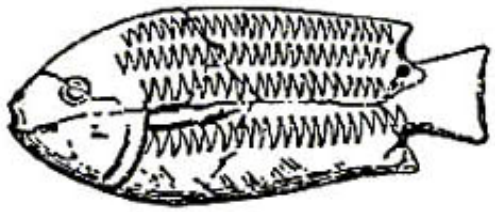

Fig. 9 Tilapia amulet form the Tomb of Mena, First Dynasty of ancient Egypt.

Male Nile tilapias are polygynous, they build and defend spawning pits where they mate with a number of females. All *Oreochromis* species are maternal mouthbrooders. After fertilization, the females pick the eggs and incubate them in their buccal cavity. Subsequently, yolk-sac fry stays in the mouth of the mother until the start of external feeding. At this time, fry is guarded by the mother and in case of danger sheltered in the mouth (Beveridge, 2000). Females can spawn 4-6 times per year and a single female produces 1-2 eggs per gram of body.

Cichlids have been studied for a long time especially for their remarkable evolutionary radiation and diversity of Haplochromini flocks in the Great Lakes of Africa. As the commercial value for tilapia cichlids increased, they have been extensively investigated in the field of endocrinology, molecular genetics and reproduction. *Oreochromis niloticus* proved to be very suitable model species for biological studies with many advantages. The fish are easy to rear in the aquarium. They can be bred year-round with a short generation period (they

reach sexual maturity in five to six months). They have large eggs, embryos and larva, which makes them useful for various observations. Adult fish are also large and therefore suitable for diverse physiological studies. At present, almost 6000 entries for tilapia can be found throughout nucleotide database at NCBI, sequencing of the tilapia genome is underway by The Cichlid Genome Consortium (http://hcgs.unh.edu/cichlid/). There are successful transgenic technologies applied for Nile tilapia (Rahman and Maclean, 1992; Pohajdak et al., 2004).

Aims of the study

Similarly to mammals, after birth liver becomes the major source for circulating IGF-I in fish (Reinecke and Collet, 1998). However, the actions of IGF-I in different non-hepatic tissues are thought to be complemented by locally produced IGF-I, that acts in autocrine or paracrine fashion. In adult teleost fish, IGF-I is produced in numerous organs such as: central nervous system, gills, muscles, gastro-intestinal tract, kidney, heart and gonads (Reinecke et al., 1997; Perrot et al., 1999; Maures et al., 2002; Vong et al., 2003). Also in the tilapia, in addition to liver, IGF-I mRNA transcripts have also been reported to occur in various extra-hepatic sites (Caelers et al., 2004). The local action of IGF-I may even be more important during early development. The expression of IGF-I during fish ontogeny has been reported, mainly focusing on IGF-I content in the whole fish body (Greene and Chen, 1999b; Perrot et al., 1999; Ayson et al., 2002; Maures et al., 2002; Deane et al., 2003; Gabillard et al., 2003), whereas the tissue distribution of IGF-I and its cellular localization during fish ontogeny has been described so far only in few reports (Berwert et al., 1995; Perrot et al., 1999; Radaelli et al., 2003).

This study investigates the presence of IGF-I during early ontogeny of tilapia, during the processes of organogenesis, somatic growth and sexual differentiation. The aim is to understand the possible pathways of IGF-I action looking at the endocrine, liver-derived route, as well as at local non-hepatic IGF-I. For this purpose, a morphological approach, using the correlation of immunohistochemistry and *in situ* hybridization techniques is employed to reveal the cellular localization of IGF-I peptide and mRNA, respectively. The study is carried out on monosex fry of Nile tilapia, *Oreochromis niloticus*, during its development and in adult life.

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Chapter 1

Organ-specific expression of IGF-I during early development of bony fish as revealed in the tilapia, *Oreochromis niloticus*, by in situ hybridization and immunohistochemistry: indication for the particular importance of local IGF-I*

The cellular sites of insulin-like growth factor I (IGF-I) synthesis in the early developing tilapia (0-140 days post fertilization, DPF) were investigated. IGF-I mRNA and peptide appeared in liver as early as 4 DPF and in gastro-intestinal epithelial cells between 5-9 DPF. In exocrine pancreas, the expression of IGF-I started at 4 DPF and continued until 90 DPF. IGF-I production was detected in islets at 6 DPF in non-insulin cells and occurred throughout life. In renal tubules and ducts, IGF-I production started at 8 DPF. IGF-I production in chondrocytes had its onset at 4 DPF, was more pronounced in growing regions and was also found in adults. IGF-I mRNA and peptide appeared in the cytoplasm of skeletal muscle cells at 4 DPF. In gill chloride cells, IGF-I production started at 6 DPF. At 13 DPF, IGF-I was detected in cardiac myocytes. IGF-I-producing epidermal cells appeared at 5 DPF. In brain and ganglia, IGF-I was expressed in virtually all neurones from 6 to 29 DPF, their number decreasing with age. Neurosecretory IGF-I-immunoreactive axons were first seen in the neurohypophysis around 17 DPF. Endocrine cells of the adenohypophysis exhibited IGF-I mRNA at 28 DPF and IGF-I immunoreactivity at 40 DPF. Thus, IGF-I appeared early (4-5 DPF), first in liver, the main source of endocrine IGF-I, and then in organs involved in growth or metabolism. The expression of IGF-I was more pronounced during development than in juvenile and adult life. Local IGF-I therefore seems to have a high functional impact in early growth, metabolism and organogenesis.

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Introduction

Autocrine, paracrine and endocrine signals co-ordinate the direction of differentiation of tissues during critical periods of development. The differentiation of organs thus involves a complex cascade and interaction of signals whose organisational action on tissue differentiation is dependent on being released at precise times, at correct sites (in the case of para- and autocrine factors) and within a specific dose range (Segner at al. 1994). Insulin-like growth factor I (IGF-I) plays a central role in the complex system that regulates growth, differentiation, and reproduction (see review: Reinecke and Collet 1998).

Like in mammals, the major site of IGF-I gene expression in bony fish is liver but several extrahepatic sites also express IGF-I (see reviews: Reinecke and Collet 1998, Duan 1998¸ Reinecke et al. 2005; Wood et al. 2005). Recently, by the use of real-time reverse transcription/polymerase chain reaction (RT-PCR), the absolute amounts of IGF-I were measured in adult tilapia, *Oreochromis niloticus* (Caelers et al. 2004), and significant levels of IGF-I mRNA were measured not only in liver but also in other organs, such as brain, gills, heart, intestine, kidney, skeletal muscle, spleen and testes. These results are consistent with the earlier immunohistochemical identification of parenchymal cells as local production sites in numerous organs of tilapia (Reinecke et al. 1997) and indicate paracrine/autocrine actions of local IGF-I involved in organ-specific functions of adult bony fish.

Some studies in bony fish showing the presence of IGF-I and its receptor (IGF-1R) in fish embryos suggest a high organisational impact of IGF-I during ontogeny. Developmental and tissue specific regulation of the steady-state mRNA levels of the IGF-1R and polyadenylation were detected in rainbow trout (Greene and Chen 1999) and seabream (Perrot et al. 1999). Correspondingly, some RT-PCR studies on trout (Shamblott and Chen, 1993; Duan et al. 1995; Greene and Chen, 1997, 1999), seabream (Duguay et al. 1996; Perrot et al. 1999) and rabbitfish (Ayson et al. 2002) suggest that the expression patterns of IGF-I mRNA in several organs are also age-dependent. The physiological importance of IGF-I in early fish development is also stressed by the demonstration of marked mitogenic effects of IGF-I on zebrafish embryonic cells (Pozios et al. 2001).

However, little information exists regarding the cellular sites of IGF-I synthesis, with only two studies in seabream (Perrot et al. 1999) and rabbitfish (Radaelli et al. 2003) and

some preliminary data in tilapia (Shved et al. 2005) reporting the localisation of IGF-I during ontogeny. These reports involve the investigation of several organs at different larval stages by the use of immunohistochemistry. However, no thorough study on the cellular IGF-I production during early fish development has been performed and no other species have been studied. Furthermore, to date, in situ hybridization has not been used to detect the cellular expression of IGF-I mRNA during ontogeny. Therefore, we have investigated the potential production sites of IGF-I in numerous organs, such as liver, gastro-intestinal tract, exo- and endocrine pancreas, kidney, skeletal muscle, cartilage, gills, skin, brain and pituitary, in early developmental stages, i.e. from 0 day post fertilization (DPF) to 140 DPF, of tilapia (*Oreochromis niloticus*) and in adult individuals. The study uses immunohistochemistry to localize the IGF-I peptide and in situ hybridization for the expression of IGF-I mRNA.

Material and methods

Animals

Fry and adult *Oreochromis niloticus* used in this study originated from the Aquaculture Experimental facilities of CIRAD (Montpellier). Fertilized eggs were either obtained through natural or artificial fertilizations. For natural fertilizations, *0. niloticus* breeders (1 male and 3 females) were maintained in a spawning aquarium of 360 l maintained at constant temperature (27°C) and photoperiod (12L:12D). Under natural photoperiods, reproductive activity occurred mainly from the afternoon until sunset in this species (Baroiller et al. 1997). Consequently, we checked every morning for the presence of newly incubating females, which were then isolated from the other fish. On the first day after fertilization, eggs were gently removed from the mouth of the female, and incubated in 1-l McDonald jars at 27 ± 1 °C (mean range). For artificial fertilizations, maturation of isolated females was carefully recorded (i.e. uro-genital papilla development and behavior). When nearly breeding stage was reached, both male and female breeders were striped under anesthesia. Fertilizations were performed according to Chourrout and Itskovitch (1983). After fertilization, eggs were incubated in 1-l McDonald jars, maintained at 27°C, to the free-swimming stage (around 10

DPF). Just before the completion of yolk sac absorption, fry were placed into a 50-l tank in an indoor recirculating system at 27±1°C. They were fed with commercial salmonid food.

Fish larvae at different developmental stages, i. e. from 0-140 DPF, and adults were sampled and anaesthetized by the addition of 2-phenoxy-ethanol (Sigma, St. Louis, MO, USA) to water (0.3 ml/l). For the earliest stages, trunk was cut to allow fixation solution to enter. If the larvae had reached a sufficient size, the organs were excised. Tissue preparations were fixed with Bouin's solution without acetic acid for 4 h at room temperature. Specimens were dehydrated in an ascending series of ethanol and routinely embedded in paraplast (58°C).

Generation of tilapia-specific probes for in situ hybridization

Probes used were prepared as already described (Schmid et al. 1999). In brief, total RNA from tilapia liver was extracted by the phenol/chloroform method with the Ultraspec Extraction Kit (ams, Lugano, Switzerland). For cDNA synthesis, 5 µg RNA were annealed with 1 µM of a poly(dT) primer (5' CCT GAA TTC TAG AGC TCA T(dT17) 3') for 3 min at 70°C. The RNA/primer mix was incubated for 1 h at 37°C with 15 mM dNTPs and 10 U AMV-reverse transcriptase (Pharmacia, Switzerland) in 1x reaction buffer (50 mM TRIS-HCl pH 8.3, 40 mM KCl, 6 mM $MgCl_2$). A 1-µl cDNA aliquot was incubated with 1 µM of the sense (5`-GTC TGT GGA GAG CGA GGC TTT-3`) and antisense primer (5'-AAC CTT GGG TGC TCT TGG CAT G-3`), corresponding to the B- and E- domains (Reinecke et al. 1997*),* 200 µM dNTPs, and 1 U *Taq*-polymerase (Pharmacia) in 1x incubation buffer (10 mM TRIS-HCl pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatine). The amplification program was optimized for a Stratagene RoboCycler Gradient 40 as follows: one cycle of 10 min at 94°C, 1 min at 59°C, 2 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 59°C and 2 min at 72°C followed by a final extension step of 5 min at 72°C. PCR fragments were separated on a 2 % agarose gel and eluted by the Gel Extraction Kit QIAquick (Qiagen, Switzerland). PCR products were cloned in a pCR-Script SK(+) cloning vector (Stratagene, Heidelberg, Germany). After propagation of *Escherichia coli* containing the plasmids, purification of the plasmids was performed with the Midi Purification Kit (Qiagen). Plasmids with the IGF-I fragment were sequenced (Microsynth, Switzerland) and the sequences were compared with those in a database. The plasmids containing the specific inserts of IGF-I (207

bp), were used as templates for the synthesis of the digoxigenin (DIG)-labelled RNA probes. Linearization was performed with the restriction enzyme EcoRI for T3 polymerase-driven transcription and with NotI for T7 polymerase-driven transcription. After ethanol precipitation, linearization efficiency was assured on a 1.2% agarose gel. The linearized plasmids (1 µg) were transcribed in vitro by using a transcription kit (Roche Diagnostics, Germany) in the presence of DIG-UTP to obtain the antisense and sense probes. The IGF-I probes selectively detected tilapia IGF-I and not tilapia IGF-II, as shown previously (Schmid et al. 1999*)*. Integrity of probes and efficiency of labelling were confirmed by gel electrophoresis, including blotting and incubation with antibody (Ab), and by dot blot. For dot blot, 1 µl of different dilutions (undiluted, 1:10, 1:100, 1:1000) of probes and control RNAs were dropped on a nylon membrane (Roche-Diagnostics) and fixed by UV cross-linking (254 nm, 125 mJ). The membrane was washed with 1% blocking reagent (Roche-Diagnostics) in buffer P1 for 30 min. After a rinse with P1 the membrane was equilibrated with buffer P3 and finally washed with 1:50 diluted nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) stock solution (Roche-Diagnostics) until colour development. For gel electrophoresis and blotting probes (200 ng) were loaded on a 1% denaturing formaldehyde agarose gel in 1x MOPS and run at 80 V for 1.5 h. After the run, the gel and the membrane were shaken in diethylpyrocarbonate (DEPC)-H_20 for 5 min. Capillary transfer was carried out with 20x standard sodium citrate (SSC) overnight. Nucleic acids were fixed by UV-crosslinking. Subsequently, the membrane was incubated with AP-labelled anti-DIG Ab by applying the same procedure as for dot blot. Lengths of the probes were compared with a control RNA (760 nt, Roche Diagnostics).

In situ hybridization protocol

Sections (4 µm thick) were mounted on Super Frost Plus slides (Menzel-Gläser, Germany) and dried overnight at 42°C. After being dewaxed and rehydrated, the sections were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 1x phosphate-buffered saline (PBS). The following steps were carried out with DEPC-treated solutions in a humified chamber. The sections were digested with 0.02% proteinase K in 20 mM TRIS-HCl pH 7.4, 2 mM $CaCl_2$ for 10 min at 37°, treated with 1.5 % triethanolamine and 0.25% acetic anhydride for 10 min at room temperature and incubated with 50 µl prehybridization solution per section for 3-4 h at

54°C. Hybridization was carried out overnight at 54°C with 30 µl of hybridization buffer containing 10 ng of sense or antisense probe previously denaturated for 5 min at 85°C. Slides were washed for 15 min at room temperature in 2x SSC, and for 30 min at the specific hybridization temperature at descending concentrations of SSC (2x, 1x, 0,5x, 0,2x). The anti-DIG AP-coupled Ab was diluted 1:4000 in 1% blocking reagent (Roche-Diagnostics) in buffer P1 and applied to the sections for 1 h at room temperature in the dark. After being washed twice in P1 for 15 min, the sections were treated with buffer P3, 5 mM levamisole and NBT/BCIP stock solution (Roche Diagnostics). Colour development was carried out overnight at room temperature and stopped by rinsing in tap water for 15 min.

Immunohistochemistry

Sections were cut at 4 µm, mounted onto glass slides (Menzel-Gläser) and dried overnight at 42°C. After being dewaxed and rehydrated, they were used for immunohistochemistry. To reduce unspecific binding, sections were treated with PBS (pH 7.4) containing 2% bovine serum albumine for 30 min at room temperature. Thereafter, the sections were incubated overnight with the rabbit antiserum 116 raised against human IGF-I (Reinecke et al. 1997; Schmid et al. 1999) diluted at 1:400 and washed repetitively in PBS. The IGF-I antiserum was detected by incubation with biotinylated goat anti-rabbit IgG (Bioscience Products, Emmenbrücke, Switzerland, 1:100) for 30 min at room temperature. After repetitive rinses in PBS, the sections were incubated with streptavidin-fluorescein-isothiocyanate (Bioscience Products, 1:100) for 30 min at room temperature in the dark. Specificity of the reactions obtained was tested using the following controls: (1) replacement of the primary antiserum by non-immune rabbit serum, (2) pre-absorption of the primary antiserum with recombinant human (h) IGF-I, hIGF-II, porcine insulin (kind gift of Prof. J. Zapf, Zürich) or the peptide used for immunization (40 µg or 400 µg peptide/ml diluted antiserum). Photomicroscopy was performed with a Zeiss Axioscope using the Axiovision software 3.1. (Zeiss, Zürich, Switzerland).

Results

General

During early development, IGF-I mRNA and peptide were observed in all organs investigated. However, the onset of IGF-I expression and its duration differed considerably among the different tissues and organs (Table 1).

Liver

At 4 DPF, several hepatocytes started to exbibit IGF-I mRNA followed by IGF-I immunoreactivity around 6-7 DPF (Table 1). By 12 DPF, numerous hepatocytes showed IGF-I mRNA and some also contained IGF-I peptide (Fig. 1a,b). Around 36 DPF, a larger number of hepatocytes contained IGF-I mRNA (Fig. 1d) and about half of the hepatocytes also possessed IGF-I immunoreactivity at variable intensity (Fig. 1c). Whereas the expression of IGF-I mRNA persisted in hepatocytes, the presence of IGF-I peptide decreased with age and could not be detected after 90 DPF.

Gastro-intestinal tract

IGF-I mRNA and peptide appeared in cells of the intestinal mucosal epithelium at 5 DPF (Fig. 2 a, b). At 9 DPF, they were also detected in cells of the stomach epithelium. Virtually all epithelial cells of the gastro-intestinal tract contained IGF-I mRNA and peptide. IGF-I immunoreactive material was mainly located in the cell apex (Figs. 2a,c,d). The largest numbers of mucosal epithelial cells exhibiting IGF-I mRNA and peptide were found at 13-29 DPF (Fig. 2c-e, Table 1). Around 17 DPF, additional IGF-I containing cells were detected with the typical appearance of endocrine cells (Fig. 2c). These IGF-I immunoreactive cells occurred in all portions of the gastro-intestinal tract but their distribution and frequency varied largely among different individuals. The cells persisted until the adult stage, whereas the presence of IGF-I immunoreactivity in the cell apex was limited to about 50 DPF (Table 1).

Pancreas

In the exocrine pancreas, the first cells with IGF-I mRNA were present already at 4 DPF. Their number increased at around 6 DPF (Fig. 2f) and persisted at a high level until 18 DPF

(Table 1). Thereafter, the number of IGF-I containing acinar cells decreased until about 90 DPF and could not be detected in adults.

At 6 DPF, some islet cells containing IGF-I mRNA (Fig. 2f) and peptide. Their number increased until 18 DPF. The IGF-I containing islets cells did not contain insulin (Fig. 2g,h).

Kidney

At 8 DPF, IGF-I-immunoreactivity (Fig. 3a) and mRNA appeared in the vast majority of renal vesicles and in the pronephric ducts. Faint IGF-I immunoreactivity (Fig. 3b) and mRNA were present in the renal tubules forming around 13 DPF. In the pronephric ducts, IGF-I immunoreactivity was located in the apical cell portion (Fig. 3a,b). Around 17 DPF, the epithelial cells of the pronephric urinary ducts (Fig. 3c) contained strong IGF-I immunoreactivity in the cell apex. Furthermore, numerous proximal tubules exhibited IGF-I mRNA (Fig. 3d) and immunoreactivity (Fig. 3e), whearas most of the distal tubules showed only faint reactions (Fig. 3e). This distribution pattern persisted until 25 DPF (Fig. 3f). Later, some proximal and distal tubules contained IGF-I immunoreactivity (Fig. 3g) and mRNA. In addition, strong IGF-immunoreactivity was present in the apical cell portions of the collecting ducts (Fig. 3g). The same distribution pattern was also found in juvenile and adult tilapia (Table 1).

Cartilage

Even at 4-5 DPF, IGF-I mRNA and peptide were present in cartilage (Table 1). The expression of IGF-I was most pronounced in growing regions (Fig. 4a,b). Numerous chondrocytes expressing IGF-I were found in cartilage of various locations (Fig. 4c,d,g, 5a, 6a,d) throughout development. Their number slowly decreased with time and they were also found in adult life.

IGF-I was detected by in situ hybridisation and immunohistochemistry also in the perichondrium starting at 4 DPF. The expression of IGF-I in the perichondrium was most pronounced between 6 and 50 DPF (Table 1) and persisted throughout life at a moderate level.

Muscle

IGF-I mRNA and peptide appeared in skeletal muscle fibers at 4 DPF (Table 1). Both were present in the cytoplasm (Fig. 4 e,f). The highest expression of IGF-I in skeletal muscle was between 8 and 29 DPF (Table 1). Thereafter, it decreased (Fig. 4 g) but persisted throughout development and adult life.

Gills

The production of IGF-I in gills had its onset at 6 DPF (Fig. 5a; Table 1). From the beginning both IGF-I mRNA and peptide occurred in the majority of chloride cells. This distribution pattern was found throughout development (Fig. 5 b,c) and adult life.

Heart

At 13 DPF, IGF-I was first detected in the developing heart (Table 1). IGF-I was present both in atrial (Fig. 5d) and ventricular (Fig. 5e) myocardiocytes. The highest amount of IGF-I-containing myocardiocytes was present from 19 to 29 DPF. In general, the number of cells showing IGF-I mRNA was higher than that of cells exhibiting IGF-I-immunoreactivity (Table 1). During late development and in adults, no IGF-I could be detected in the heart.

Brain

In brain, strong IGF-I mRNA signals were revealed in numerous neurones of the brain of larvae (Fig. 6a) starting around 6 DPF (Table 1). Similarly, IGF-I immunoreactivity was present in the vast majority of neurones (Fig. 6c). In addition, IGF-I was expressed in spinal (Fig. 6d) and in developing autonomic ganglia. This overall neuronal presence of IGF-I persisted until about 29 DPF and slowly decreased thereafter. In older larvae and adult individuals, IGF-I mRNA and peptide, although still found at all levels of brain and spinal cord, were present in some neurones only. In the older fish, the number of neurones containing IGF-I mRNA exceeded that of the IGF-I-immunoreactive neurones. Whereas in most areas of the brain the distribution patterns of IGF-I mRNA and peptide varied largely among the individuals investigated, the Purkinje cells in the cerebellum always showed IGF-I peptide (Fig. 6e) and mRNA (Fig. 6f). Starting at 4 DPF, IGF-I-immunoreactivity and mRNA

were observed in the meninges (Fig. 6a). The expression of IGF-I persisted at the initial level until 50 DPF but stayed at a low level throughout life (Table 1).

Pituitary

IGF-I occurred in both portions of the tilapia pituitary but appeared later than in brain. The first neurosecretory axons containing IGF-I-immunoreactivity (Fig. 7a,c) appeared in the posterior (neuro-) pituitary around 17 DPF (Table 1). Their density decreased with age but they were still detected in adults. No IGF-I mRNA was detected in the posterior pituitary. IGF-I-immunoreactive neurons occurred in hypothalamus (Fig. 7c). In endocrine cells of the anterior (adeno-) pituitary first exhibited IGF-I mRNA at 28 DPF (Fig. 7b) followed by IGF-I immunoreactity at 40 DPF (Fig. 7c). IGF-I-immunoreactivity (Fig. 7c) and mRNA (Fig. 7d) in endocrine cells were present throughout development and in adults.

Skin

At 5 DPF, IGF-I-immunoreactivity appeared in cells of the epidermis (Fig. 8a). First, the number of epidermal cells exhibiting IGF peptide (Fig. 8b) and mRNA (Fig. 8c) increased with age to reach the highest level around 19-29 DPF (Table 1). In older larvae (Fig. 8d,e) and adults some superficial epidermal cells (arrows) and occasionally also cells in the basal epidermis (arrowhead) were IGF-I-immunoreactive or contained IGF-I mRNA (Fig. 8e).

Table 1 Semiquantitative grading of the number of parenchymal cells demonstrated by IGF-I in situ hybridization (*ISH*) and IGF-I immunohistochemistry (*IHC*)

Organ	DPF 4–5		DPF 6–7		DPF 8–12		DPF 13–18		DPF 19–29		DPF 30–50		DPF 51–70		DPF 71–90		DPF 91–140		Adult	
	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC	ISH	IHC
Brain																				
Neurones	-	-	+	+	++	++	+++	+++	+++	+++	+	+	+	+/-	+	+/-	+	+/-	+	+/-
Meninges	+	+	+	+	+	+	+	+	+	+/-	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Pituitary																				
anterior	-	-	-	-	-	-	-	-	++	-	+++	+++	+++	+++	++	++	++	++	++	++
posterior	-	-	-	-	-	-	-	++	-	++	-	+	-	+/-	-	+/-	-	+/-	-	+/-
G-I tract																				
Stomach	-	-	-	-	++	++	++++	++++	++++	++++	+++	+++	++	++	+/-	+/-	+/-	+/-	+/-	+/-
Intestine	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++	++	++	+	+	+/-	+/-	+/-	+/-	+/-	+/-
Liver	++	-	++	+	+++	+	+++	+	+++	++	+++	++	+++	+	+++	+/-	+++	-	+++	-
Pancreas																				
exocrine	+	+	++	++	++	++	++	++	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	-	-	-
endocrine	-	-	+	+	+	+	+	+	++	++	++	++	++	++	++	++	++	++	++	++
Gills	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Kidney	-	-	-	-	++	++	+++	+++	++	++	++	++	++	++	+	+	+	+	+	+
Heart	-	-	-	-	-	-	+	+	+++	+	++	+	+	+	+	+	-	-	-	-
Cartilage																				
Chondrocytes	++	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	+	+
Perichondrium	+	+	++	++	++	++	++	++	++	++	+	++	+	+	+	+	+	+	+	+
Muscle	+	+	++	++	+++	+++	+++	+++	++++	++++	++	++	++	++	++	++	+	+	+	+
Epidermis	+	+	+	+	+	+	++	++	+++	+++	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-

- none, +/- very few, + some, ++ moderate, +++ majority, ++++ overall

Table 1 Semiquantitative grading of the number of parenchymal cells demonstrated by IGF-I in situ hybridization *(ISH)* and IGF-I immunohistochemistry *(IHC)*

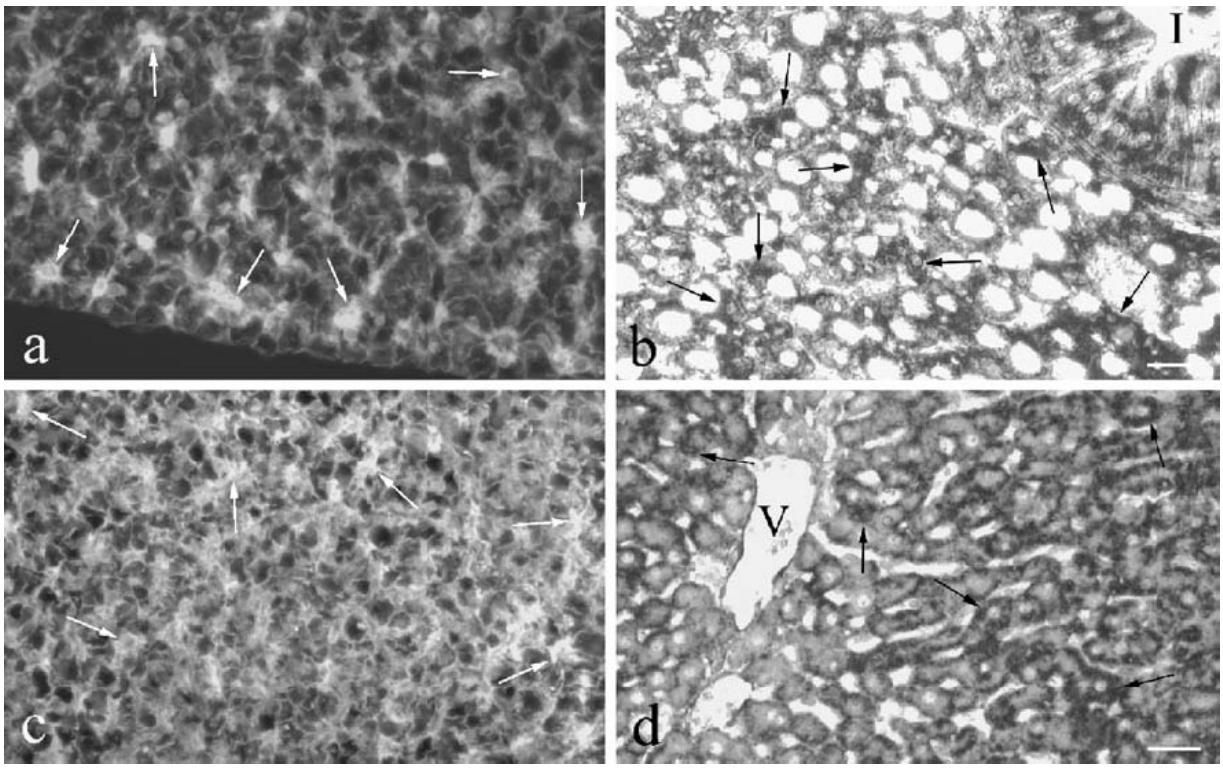

Fig. 1 IGF-I-immunoreactivity (**a, c**) and mRNA (**b, d**) in developing tilapia liver. **a, b** At 12 DPF, many hepatocytes exhibit IGF-I-immunoreactivity (**a**, *white arrows*) and IGF-I mRNA (**b**, *black arrows*). Numerous mucosal epithelial calls of the intestine (*I*) contain IGF-I mRNA (**b**). *Bar* 20 μm. **c, d** Tilapia liver at 36 DPF. IGF-I-immunoreactivity of variable intensity (**c**, *white arrows*) and mRNA (**d**, *black arrows*) occur in numerous hepatocytes (*V* vein). *Bar* 30 μm

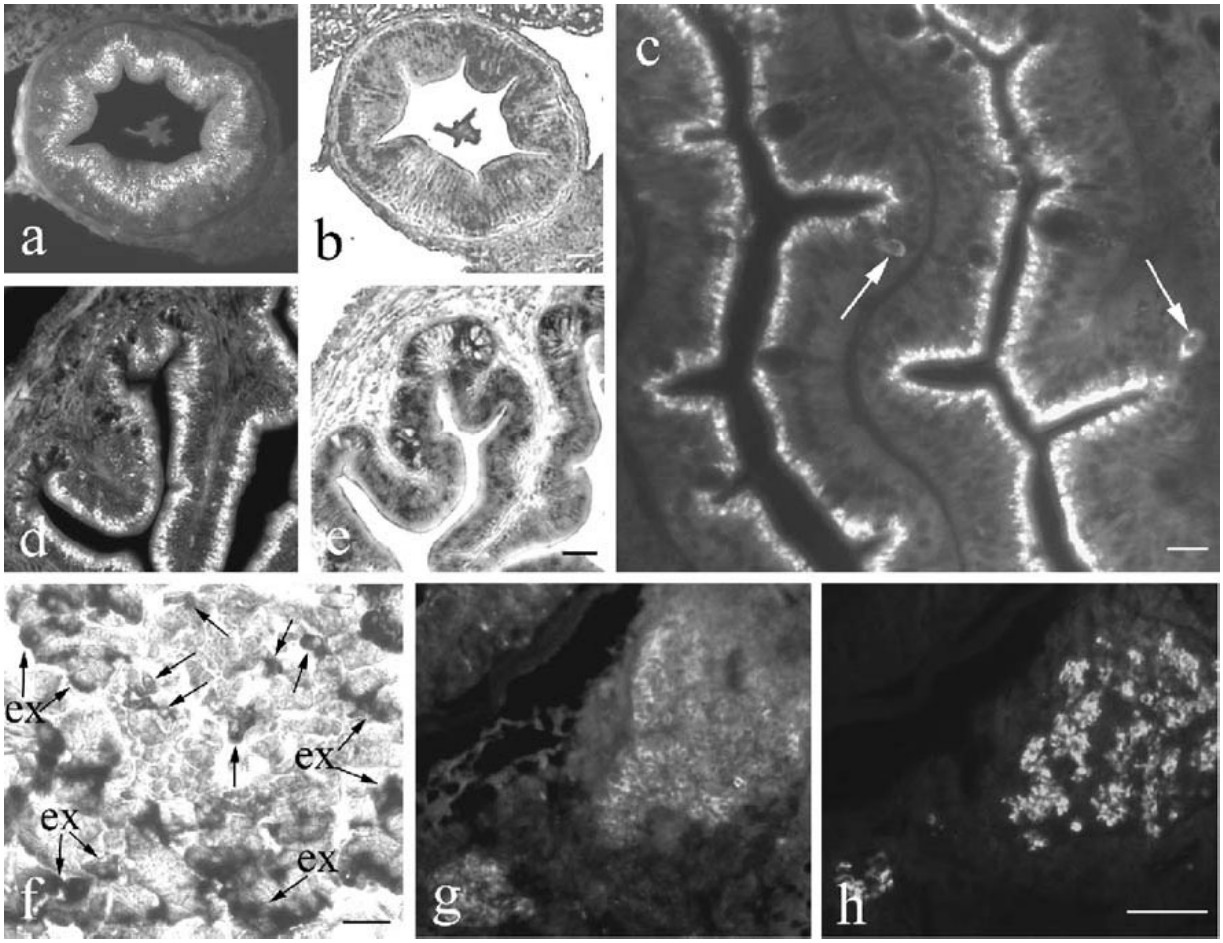

Fig. 2 IGF-I-immunoreactivity and mRNA in developing tilapia gastro-intestinal tract and pancreas. **a, b** At 5 DPF, most mucosal epithelial cells in the developing intestine show IGF-I-immunoreactivity in the apex (**a**) and also IGF-I mRNA (**b**) on consecutive sections. *Bar* 25 μm. **c** At 17 DPF, additional IGF-I containing cells (*arrows*) are found with the typical appearance of endocrine cells. *Bar* 25 μm. **d, e** At 29 DPF, numerous cells in the mucosal epithelium of the stomach are IGF-I-immunoreactive (**d**) and contain IGF-I mRNA (**e**). *Bar* 40 μm. **f** At 6 DPF, several endocrine cells in the islet contain IGF-I mRNA (*arrows*). Numerous acinar cells of the surrounding exocrine pancreas (*ex, arrows*) also exhibit IGF-I mRNA. *Bar* 25 μm. **g** At 76 DPF, no IGF-I-immunoreactivity is observed in the exocrine pancreas but numerous cells in the islets are IGF-I-immunoreactive. As shown by double immunofluorescence, these cells constitute a subpopulation different from the insulin-immunoreactive cells (**h**). *Bar* 25 μm

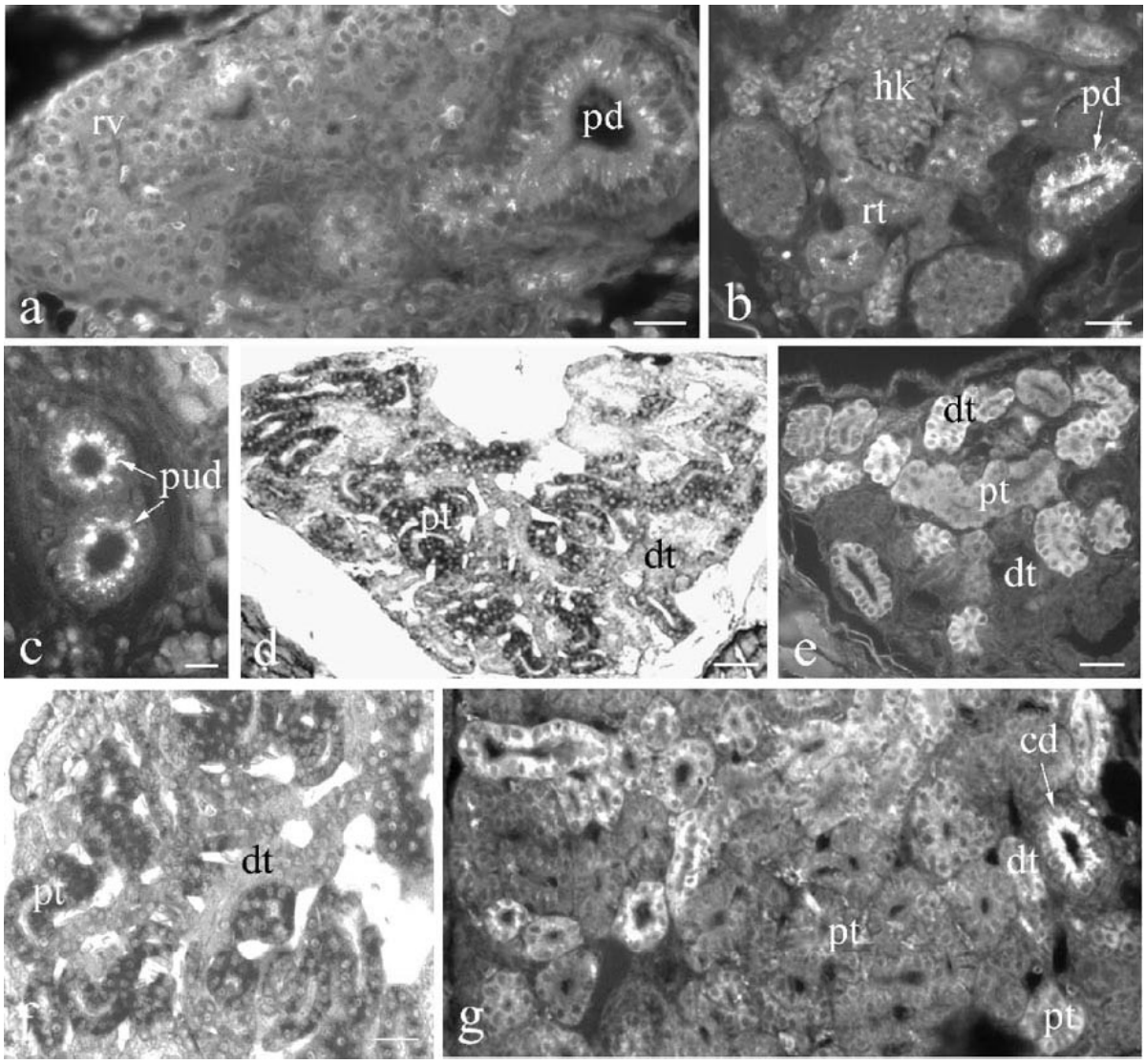

Fig. 3 IGF-I in developing kidney. **a** At 8 DPF, IGF-I immunoreactivity is present in most of the renal vesicles (*rv*) and in the pronephric duct (*pd*). Bar 25 μm. **b** At 13 DPF, several renal tubules (*rt*) contain faint IGF-I immunoreactivity. In the pronephric duct (*pd*), IGF-I immunoreactivity is located in the apical cell portion (*hk* head kidney). *Bar* 30 μm. **c-e** 17 DPF. Epithelial cells of the pronephric urinary ducts (*pud*) show strong IGF-I immunoreactivity in the apex at 17 DPF. Numerous proximal (*pt*) and distal (*dt*) tubules exhibit IGF-I mRNA (**d**) and immunoreactivity (**e**). *Bars* 20 μm (**c**), 30 μm (**d**), 20 μm (**e**). **f** At 25 DPF, most of the proximal tubules (*pt*) contain IGF-I mRNA but no expression is seen in the distal tubules (*dt*). Bar 20 μm. **g** At 29 DPF, some proximal (*pt*) and distal (*dt*) tubules as well as a collecting duct (*cd*) show IGF-I immunoreactivity. *Bar* 15 μm.

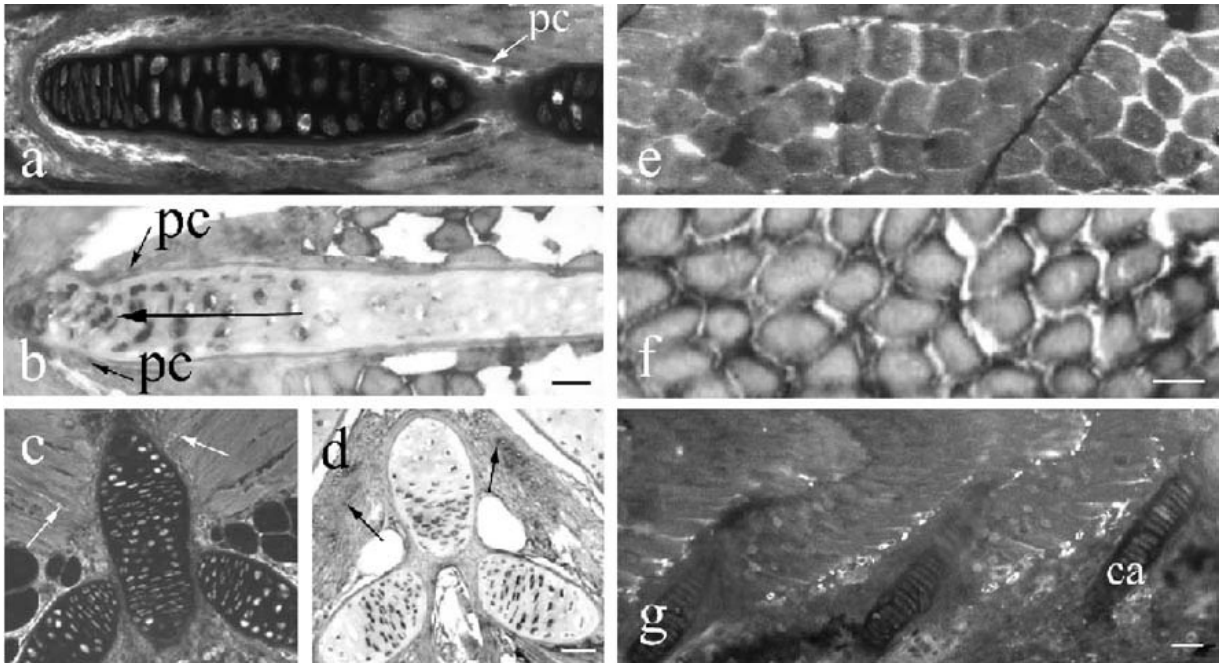

Fig. 4 IGF-I in developing cartilage (**a-d**) and skeletal muscle (**e-g**). **a** At 10 DPF, most chondrocytes and the perichondrium (*pc*) contain IGF-I-immunoreactivity. **b** The expression of IGF-I mRNA is particularly pronounced in growing regions (*large arrow*) at 17 DPF. IGF-I mRNA further occurs in the perichondrium (*pc*). *Bar* 15 μm (**a**, **b**). IGF-I immunoreactivity (**c**) and mRNA (**d**) are present in the majority of chondrocytes of spine and rib cartilage as well as in skeletal muscle (*arrows*) at 20 DPF. *Bar* 30 μm. IGF-I-immunoreactivity (**e**) and mRNA (**f**) are found in the cytoplasm of all sketetal muscle fibers at 17 DPF. *Bar* 10 μm. **g** At 50 DPF, still numerous muscle fiber show IGF-I-immunoreactivity (*ca* cartilage). *Bar* 50 μm

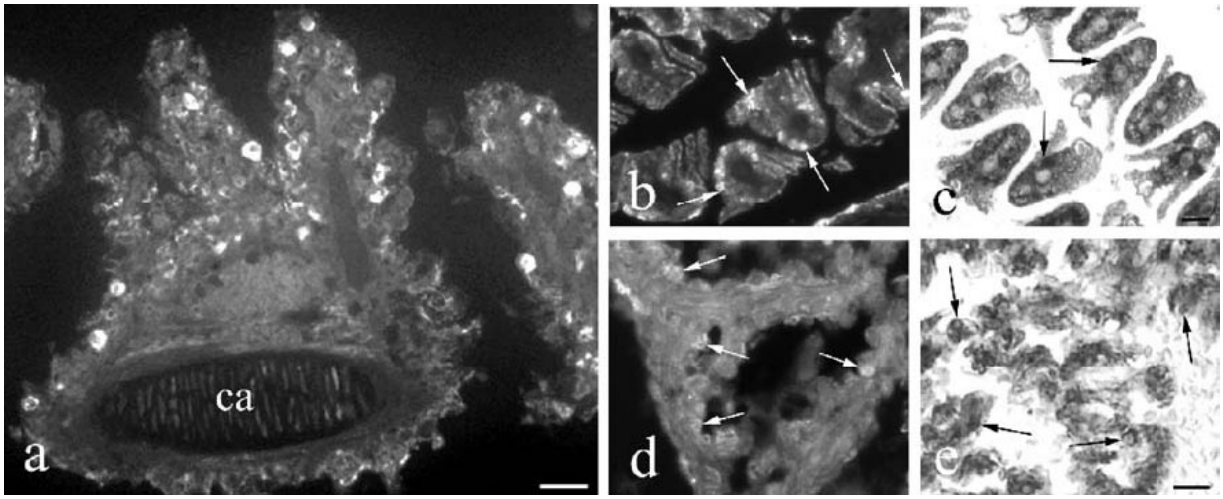

Fig. 5 IGF-I-immunoreactivity and mRNA in gills (**a-c**) and heart (**d, e**). **a** At 6 DPF, IGF-I-immunoreactivity is present in numerous of chloride cells and also occurs in gill cartilage (*ca*). Bar 45 μm. **b, c** The production of IGF-I is found in most chloride cells (*arrows*) in later development as shown for 70 DPF. *Bar* 20 μm. **d, e** The presence (*arrows*) of IGF immunoreactivity (**d**) and mRNA (**e**) in cardiomyocytes of the developing heart is shown at 29 DPF. *Bar* 20 μm

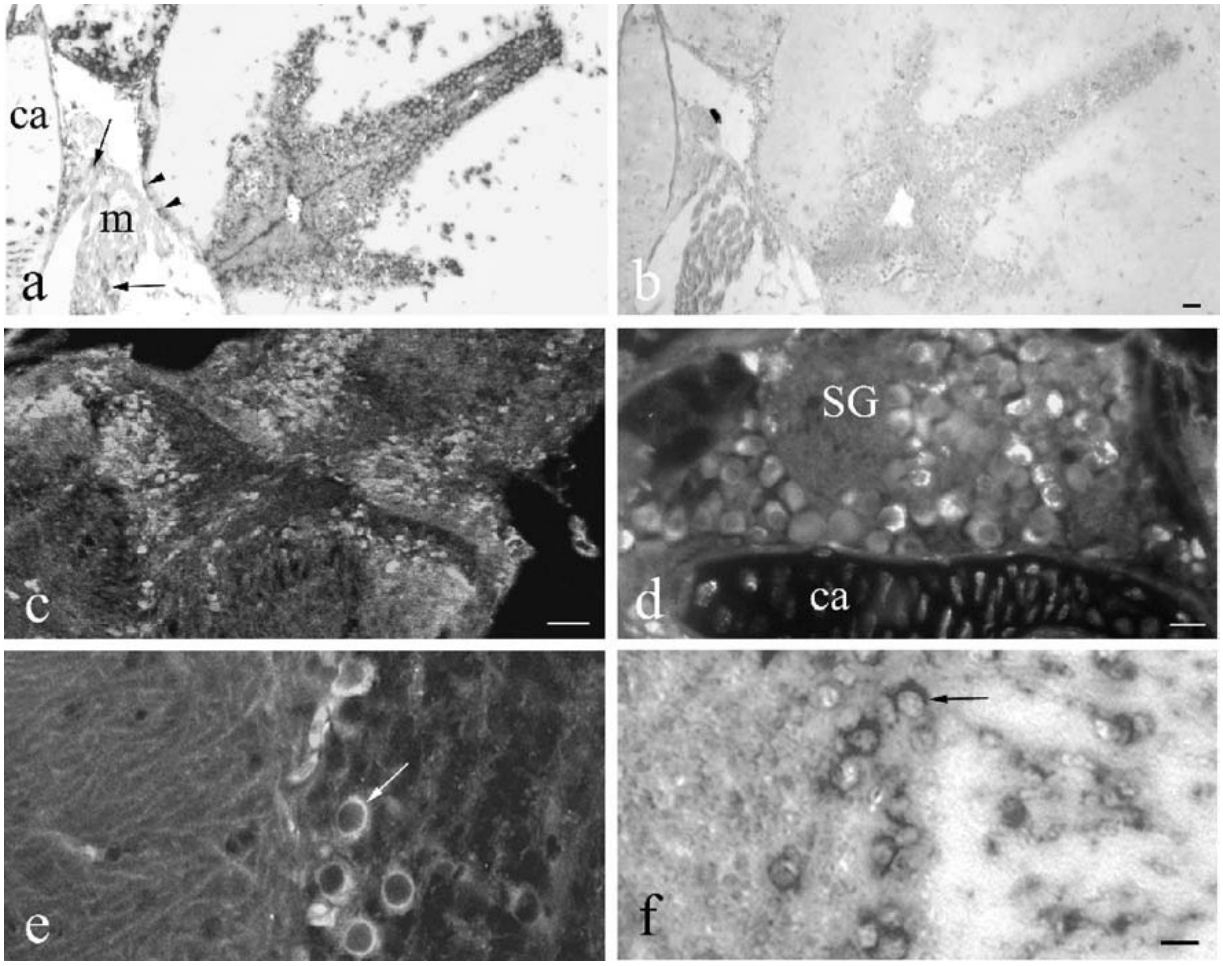

Fig. 6 IGF-I in central and peripheral nervous system. **a, b** In situ hybridisation of serial sections of tilapia brain with an IGF-I antisense probe (**a**) and an IGF-I sense probe (**b**) at 20 DPF. Abundant neurons exhibit IGF-I mRNA (**a**) while no IGF-I mRNA is located in the negative control (**b**). IGF-I mRNA also occurs in the meninges (*arrowheads*), in chondrocytes of the cartilage (*ca*) and in skeletal muscle cells (*arrows, m*). *Bar* 40 μm. **c** IGF-I-immunoreactivity is present in numerous central neurones at 20 DPF. *Bar* 100 μm**. d** Several neurones in the spinal ganglion (*SG*) exhibit IGF-I-immunoreactivity (*ca* cartilage). Bar 20 μm. **e, f** At 70 DPF, Purkinje cells (*arrows*) in the cerebellum contain IGF-I peptide (**e**) and IGF-I mRNA (**f**). Bar 25 μm

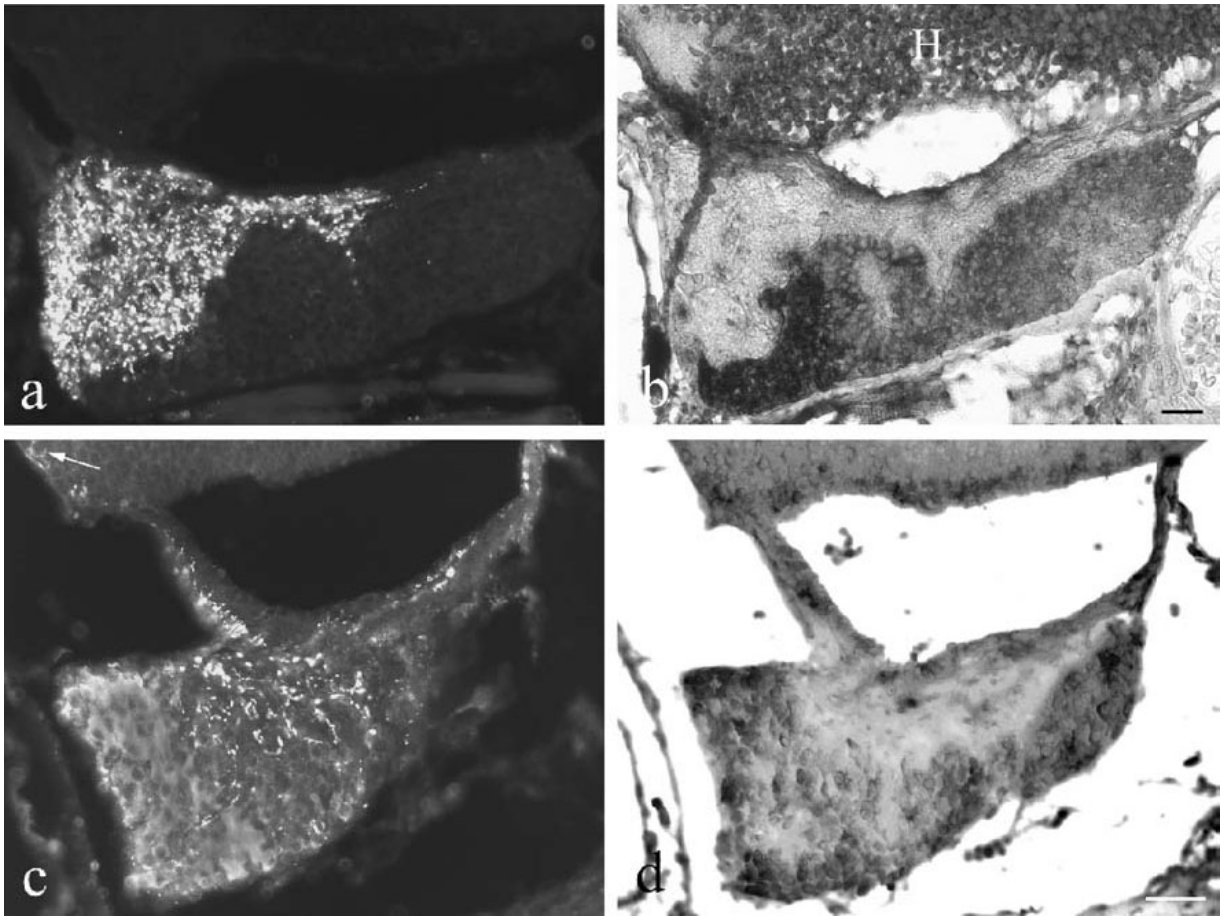

Fig. 7 IGF-I-immunoreactivity and mRNA in the pituitary as revealed on two pairs of consecutive sections (**a, b** and **c, d**) processed for immunofluorescence (**a, c**) and in situ hybridisation (**b, d**). **a, b** At 28 DPF, numerous axons in the neuropituitary contain IGF-I immunoreactivity (**a**) but no IGF-I mRNA is present (**b**). In the adenopituitary, most of the endocrine cells exhibit IGF-I mRNA (**b**), whereas they do not show IGF-I-immunoreactivity (**a**). Most neurons of the hypothalamus (*H*) exhibit IGF-I mRNA (**b**). *Bar* 40 μm. **c, d** At 40 DPF, both IGF-I immunoreactivity **(c**) and mRNA (**d**) are detectable in endocrine cells of the anterior pituitary. IGF-I-immunoreactive axons are present in the posterior part. An IGF-I-immunoreactive neuron (**c**, *arrow*) is shown in the hypothalamus. *Bar* 70 μm

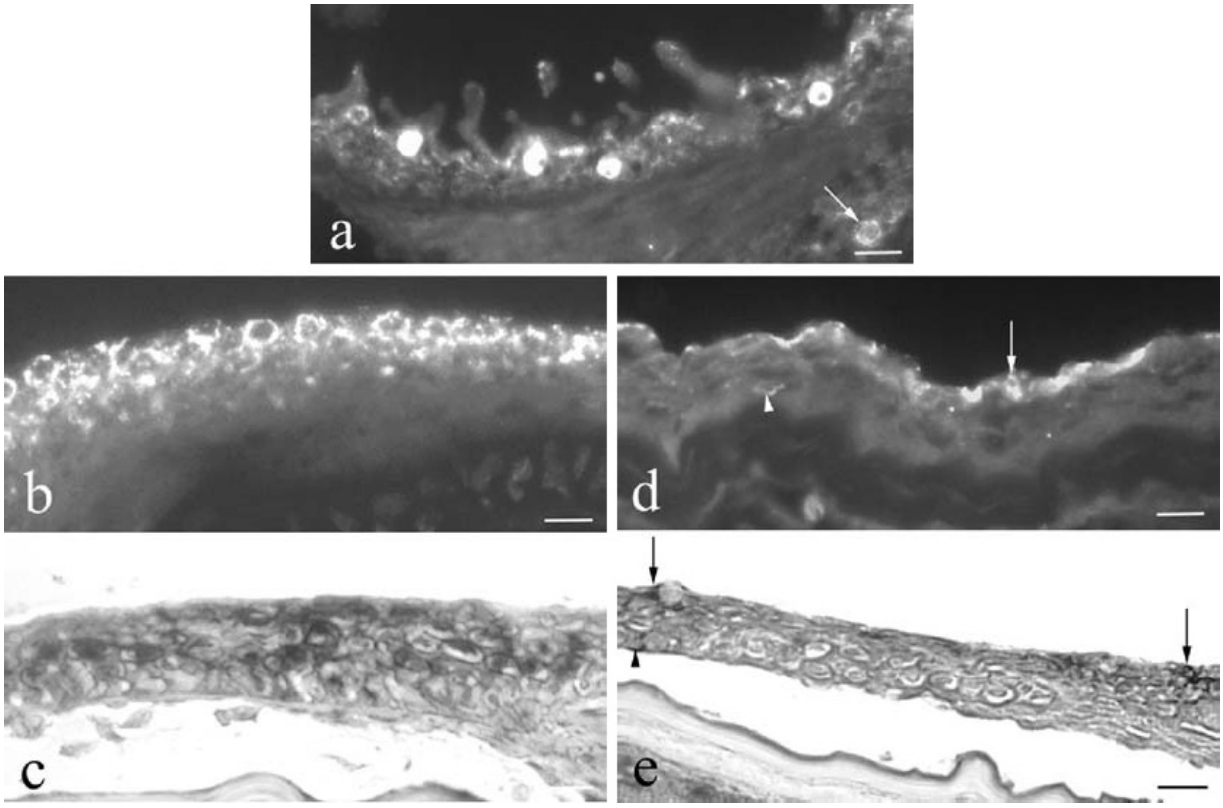

Fig. 8 IGF-I-immunoreactivity and mRNA in the skin. **a** At 5 DPF, several cells in the epidermis contain IGF-I-immunoreactivity. In the dermis, a possible dendritic cell also shows IGF-I-immunoreactivity (*arrow*). Bar 40 μm. **b, c** At 25 DPF, numerous cells in all epidermal layers exhibit IGF-I peptide (**b)** and mRNA (**c**). Bar 30 μm. **d, e** Frequent superficial epidermal cells (*arrows*) and infrequent basal epidermal cells (*arrowheads*) exhibit IGF-I immunoreactivity (**d**) and mRNA (**e**) at 36 DPF. *Bars* 50 μm (**d**), 40 μm (**e**)

Discussion

The study shows the appearance and distribution of IGF-I mRNA and peptide during early development of the tilapia, *O. niloticus*. Both IGF-I mRNA and peptide were observed in parenchymal cells of all organs investigated but the onset, intensity and duration of IGF-I expression differed considerably among the various tissues and organs.

Both IGF-I mRNA and peptide appeared in liver at 4 DPF. This early onset of IGF-I production in tilapia liver is in agreement with results obtained in shi drum where IGF-I immunoreactivity was detected in hepatocytes during the first week post hatching (Radaelli et al. 2003). Because liver is the main source of endocrine IGF-I, these results suggest a particular physiological impact of circulating IGF-I in early development. Although high expression of IGF-I mRNA was detected throughout life in tilapia liver, IGF-I-immunoreactivity decreased with age and was present only until about 70 DPF. In contrast to an earlier study on sea bream (Funkenstein et al. 1997), an intense response by in situ hybridisation for IGF-I was obtained also in adult tilapia liver. In spite of the clear expression of IGF-I mRNA in adult tilapia liver (Schmid et al. 1999), no IGF-I-immunoreactive hepatocytes could be identified in adult barramundi (Richardson et al. 1995) and tilapia (Reinecke et al. 1997; Schmid et al. 1999). Similarly, in rat liver, IGF-I-immunoreactivity was localised only after pretreatment with colchicine (Hansson et al. 1988). Therefore, IGF-I might be released from fish liver into the circulation immediately after synthesis, as has previously been presumed in mammals (Hansson et al. 1988).

High-affinity hepatic-binding sites for growth hormone (GH) have been identified in the liver of several fish species, including tilapia (Ng et al. 1992). In accordance, there is strong evidence that GH stimulates IGF-I expression in fish liver and its secretion into the circulation (see Reinecke et al. 2005). Injection or oral administration of GH significantly enhanced the IGF-I mRNA level in the liver of numerous species (e.g. Duan et al. 1993; Duguay et al. 1994, 1996; Moriyama 1995; Shamblott et al. 1995; Shepherd et al. 1997; Vong et al. 2003; Biga et al. 2004). The GH-induced increase in liver IGF-I mRNA expression is accompanied by an increase in the level of circulating IGF-I (Funkenstein et al. 1989; Niu et al. 1993; Moriyama et al. 2000). In addition to these in vivo studies, some in vitro investigations have also demonstrated the stimulating effect of GH on liver IGF-I mRNA

expression, i.e. on primary cultured hepatocytes of salmonids (Duan et al. 1993; Shamblott et al. 1995, Pierce et al. 2005) and tilapia (Schmid et al. 2000). In rainbow trout, GH mRNA was detected in early stage embryos (Yang et al. 1999). Thus, production and release of liver IGF-I during tilapia early development may be under the control of GH.

The early onset of the production of IGF-I in tilapia gastro-intestinal tract, i.e. at 5 DPF in intestine and at 9 DPF in stomach, supports the results of earlier studies. In the intestinal epithelium of turbot (Berwert et al. 1995) and shi drum (Radaelli et al. 2003), the first IGF-I-immunoreactive cells can be found as early as the first week post hatching. Furthermore, the IGF-1R appeared in the second week post hatching in shi drum gut (Radaelli et al. 2003). The number of IGF-I producing cells in the developing bony fish gastro-intestinal tract by far exceeds that in the adult because, during ontogeny, almost all epithelial cells throughout the gastro-intestinal tract exhibited IGF-I mRNA and peptide. The IGF-I immunoreactive material is mainly located in the cell apex as has also reported in shi drum (Radaelli et al. 2003), possibly suggesting release into the lumen. Around 17 DPF, additional infrequent IGF-I containing cells appear in the mucosal epithelium; these cells have the typical appearance of endocrine cells as characteristic for adult fish (Reinecke et al. 1997, Koppang et al. 1998). The distribution of these IGF-I immunoreactive cells in the various portions of the adult gastro-intestinal tract as well as their frequency vary largely among different individuals, as prevously reported in turbot (Berwert et al. 1995) and Atlantic salmon (Koppang et al. 1998),

Although the early and overall appearance of IGF-I and its receptor strongly suggest a particular physiological impact of IGF-I in the developing gastro-intestinal tract, we can only hypothesize on the physiological meaning of intestinal IGF-I in fish. IGF-I secreted from the IGF-I-immunoreactive epithelial cells may exert mitogenic functions by acting on neighbouring epithelial cells. This hypothesis is supported by results obtained in mammals. In pig, the amounts of intestinal IGF-I-immunoreactivity and of the IGF-1R can be correlated with villous growth and maturation (Schober et al. 1990) and, in adult rat, IGF-I potently stimulated crypt cell proliferation and villus cell density (Steeb et al. 1994). The potential proliferative action of intestinal IGF-I during development and adult life may be regulated by GH, which markedely increases the amount of intestinal IGF-I mRNA in juvenile common carp (Vong et al. 2003) and adult rainbow trout (Biga et al. 2004).

In juvenile and adult bony fish, IGF-I released from the unfrequent IGF-I-immunoreactive mucosal epithelial cells may exert paracrine effects in response to altering local demands, such as repair. In agreement with this hypothesis, the therapeutical potential of IGF-I has been discerned for numerous acute bowel disorders resulting in accelerated intestinal repair and epithelial regrowth (Howarth 2003). Because, in addition to gill and kidney, the intestinal tract exerts important osmoregulatory functions in fish, intestinal IGF-I may also be involved in osmoregulation (Koppang et al. 1998; Reinecke and Collet 1998). This hypothesis is supported by experiments in brown trout suggesting that IGF-I plays a role in the regulation of intestinal Na^+,K^+-ATPase activity (Seidelin and Madsen 1999).

IGF-I-immunoreactivity has been localized in cells of the exocrine parenchyma of shi drum and seabream larvae (Funkenstein et al. 1997; Perrot et al. 1999; Radaelli et al. 2003); this is supported by the results of the present study. As IGF-I production in pancreas is stimulated by GH in rat (Jevdjovic et al. 2004) IGF-I in fish pancreas may be also under the control of GH although no results have been presented to date on the potential regulation of IGF-I in the exocrine pancreas.

To date, indications for the presence of IGF-I in the endocrine pancreas have only been obtained by immunohistochemical studies. These have shown that IGF-I immunoreactivity occurs in islet cells of several bony fish species (Reinecke et al. 1993, 1997; Berwert et al. 1995; Richardson et al. 1995). The present study provides the additional information that IGF-I mRNA is also present in islet cells. This is supported by molecular biological studies that have shown that IGF-I mRNA expression in principal islets of the salmon *O. gorbusha* (Plisetskaya et al. 1993) and of *Cottus scorpius* (Loffing-Cueni et al. 1998). Islet IGF-I appeared at 6 DPF in tilapia (this study) and day 10 post hatching in turbot (Berwert et al. 1995) suggesting a significant physiological role for islet-derived IGF-I during larval development. In both species, from the onset of its expression, IGF-I occurs in non-insulin cells. This observation is in agreement with studies on adult teleosts, such as eel, tilapia, goldfish, turbot and common carp (Reinecke et al. 1993, 1997; Berwert et al. 1995).

On the one hand, islet-derived IGF-I may be involved in the paracrine regulation of insulin secretion from the β-cells although the evidence in mammals is conflicting (Leahy and Vandekerkhove 1990; Van Schravendijk et al. 1990; Jevdjovic et al. 2004). On the other hand, some studies suggest that islet-derived IGF-I may act as an endocrine hormone.

Hypophysectomy does not influence the amount of sulphation activity in eel pancreas (Duan and Hirano 1992) suggesting that islet IGF-I is not regulated by GH. In goby, islectomy leads to a decrease in $^{35}SO_4$-incorporation in cartilage (Bern et al. 1991) but hepatic GH binding was unchanged (Kelley et al 1993). The observed effects may have been raised by insulin (Plisetskaya 1998). However, in addition to the GH-dependent liver IGF-I system, islet-derived IGF-I constitutes a further endocrine GH-independent IGF-I system involved in regulation of fish growth.

No information is available on IGF-I in developing fish kidney. In adult bony fish, however, epithelial cells mainly of the proximal tubules have been reported as sites of IGF-I production in tilapia (Reinecke et al. 1997), as has similarly been described in adult mammals (see: Reinecke and Collet 1998). In tilapia, IGF-I occurred in renal vesicles even at early stages of development, i.e. at 5 DPF. Later, it was present in proximal and distal tubules and in collecting and urinary ducts. Whereas IGF-I in the tubular sytem is detected throughout the cytoplasm, it occurs only in the apical cell part in the duct possibly suggesting the release of IGF-I into the lumen. Apart from gills, kidney is a major osmoregulatory organs in fish but the evidence for a potential participation of renal IGF-I in osmoregulation is conflicting. In rainbow trout, transfer to seawater has been reported to increase the IGF-I mRNA level in kidney (Sakamoto and Hirano 1993). In contrast, the same treatment did not affect the Na^+,K^+-ATPase gene level and activity in brown trout (Madsen et al. 1995) and the renal IGF-I mRNA level in the four-spine sculpin, *Cottus kazika* (Inoue et al. 2003). Thus, at present we can only speculate about the physiological role of IGF-I in bony fish kidney. In addition to its possible role in osmoregulation, renal IGF-I may be involved in several other parameters of kidney function, such as in the stimulation of kidney growth and differentiation, renal blood flow, glomerular filtration rate and sodium absorption, as is likely in mammals (Hirschberg 1996).

In *O. niloticus*, IGF-I also appears early in tissues that are highly involved in growth, such as cartilage and skeletal muscle. At 4 DPF, IGF-I mRNA and peptide are present in the chondrocytes of cartilage of various location. Similarly, in shi drum IGF-I immunoreactivity has been observed in cartilage at day 11 post hatching (Radaelli et al. 2003). The expression of IGF-I in tilapia is most pronounced in growing regions. The number of IGF-I containing chondrocytes is higher during development than in adult life, thus underlining the

physiological impact of local IGF-I during cartilage growth. Although the GH/liver IGF-I axis is involved in the regulation of fish growth as endocrine system (Duan 1998; Reinecke et al. 2005) an influence of GH on growth via local auto/paracrine IGF-I, as indicated by the present results, is also likely. An early study on eel has determined that the stimulatory effect of GH on sulfate incorporation into cartilage is mediated by an IGF-like plasma factor (Duan and Inui, 1990). In agreement, the injection of GH into coho salmon cartilage stimulates sulfate and thymidine incorporation (McCormick et al. 1992; Tsai et al. 1994). Thus, GH may have increased both uptakes via stimulation of local IGF-I, because IGF-I peptide and mRNA have been shown in chondrocytes of developing (Perrot et al. 1999; Radaelli et al. 2003; this study) and adult (Funkenstein et al. 1997; Reinecke et al. 1997) bony fish. The GH-dependent growth promoting effects of IGF-I on cartilage may therefore be exerted not only via the endocrine route, but also in an autocrine/paracrine manner by IGF-I released from local chondrocytes, as it has been shown in rat (Reinecke et al. 2000).

In contrast to skeletal muscle of young shi drum larvae (Radaelli et al. 2003) in which no IGF-I immunoreactivity has been detected, the onset of IGF-I production in tilapia skeletal muscle occurs at 4 DPF, with IGF-I showing a maximum around 10-29 DPF, decreasing afterwards but persisting throughout life. In agreement with an auto/paracrine function of muscle IGF-I, IGF-I receptor number and binding in trout skeletal muscle are highest at 5 weeks and both parameters decrease with age (Mendez et al. 2001). Furthermore, a parallel age-related decline has been found for tyrosine kinase activity for the IGF-1R (Mendez et al. 2001). In rainbow trout muscle in vitro, IGF-I has been shown to be highly effective in stimulating glucose and alanine uptake into myosatellite cells whereby the degree of stimulation changed when cells differentiated to myotubes (Castillo et al. 2004). These and the present results showing that IGF-I in skeletal muscle appears already at 4 DPF and decreases with age indicate a key role for IGF-I in muscle development. IGF-I may be associated in later life with metabolism and repair mechanisms. In adult rainbow trout, the expression of IGF-I in skeletal muscle is stimulated by GH (Biga et al. 2005). Thus, GH may also regulate IGF-I production in larval musle.

Endocrine (liver-derived) IGF-I seems to have a physiological impact in smoltification. In mummichog (*Fundulus heteroclitus*; Mancera and McCormick 1998) and trout (Seidelin et al. 1999), IGF-I improves adaptation to seawater in a dose-dependent

manner. The osmoregulatory effects of IGF-I seem to be exerted directly because, in preparations of Coho salmon gills in vitro, IGF-I stimulates Na^+,K^+-ATPase (Madsen and Bern 1993). Hypophysectomy in tilapia lowers the levels of gill Na^+, K^+-ATPase when compared with sham-operated controls (Shepherd et al. 1997) indicating an involvement of the GH/liver IGF-I axis. In agreement, in a study on *Cottus kazika*, the levels of liver IGF-I mRNA hass been shown to be significantly higher in individuals reared in seawater than in those reared in freshwater (Inoue et al. 2003). In sharp contrast, a study on Coho salmon IGF-I mRNA has demonstrated that IGF-I mRNA is not significantly altered during seawater adaptation in liver but markedly increased in gills (Sakamoto and Hirano 1993). The chloride cells of filament epithelium have been shown not only to express Na^+,K^+-ATPase in trout (McCormick 1996) but also IGF-I in tilapia (this study), in shi drum larvae (Radaelli et al. 2003) and in adult tilapia (Reinecke et al. 1997). GH receptors have been detected in tilapia gills (Fryer et al. 1979) and GH treatment increases IGF-I mRNA in gills of salmonids and carp (Vong et a. 2003; Biga et al. 2004). Thus, in addition to circulating IGF-I, GH-regulated local autocrine/paracrine IGF-I in the chloride cells seems to participate in the regulation of plasma osmolality and gill Na^+,K^+-ATPase activity. Because the expression of IGF-I in tilapia gill chloride cells was detected even around 6-7 DPF, IGF-I may exert additional functions, such as influencing the growth and maintenance of the filament epithelium.

In shi drum heart, IGF-I immunoreactivity was detected in 15-day larvae (Radaelli et al 2003). Likewise, IGF-I mRNA and peptide appear at 13 DPF in tilapia. The expression of IGF-I in cardiomyocytes is clearly detectable until 70 DPF but is absent from later stages and adults indicating a particular physiological impact of IGF-I during heart development and growth. In correlation, a significant increase in IGF-1R mRNA levels (Gutierrez et al. 1995) has been observed in the rapidly growing juvenile trout heart (Greene and Chen 1999). In fish heart, local IGF-I may be even more important than circulating IGF-I. In rainbow trout heart IGF-1R mRNA levels decreased after application of GH (Biga et al. 2004) and the number of IGF-I receptors was inversely related to the concentration of circulating IGF-I (Banos et al. 1997; Moon et al. 1996).

Screening studies have identified the potential sites of IGF-I synthesis in the brains of barramundi, tilapia and sea bream. IGF-I-immunoreactive neurones are present in adult barramundi brainstem (Richardson et al. 1995) and at all levels of adult tilapia brain

(Reinecke et al. 1997). Correspondingly, IGF-I mRNA signals have been described throughout the brain of developing sea bream (Funkenstein et al 1997). The functional impact of IGF-I in brain is indicated by the presence of IGF-1R in adult carp and trout brain (Leibush et al. 1996) and by the appearence of IGF-1R-immunoreactivity in the developing brain of shi drum as early as hatching (Radaelli et al. 2003). In contrast to IGF-II (Caelers et al. 2003), the exact distribution of the IGF-I gene expression in fish brain is unknown. the IGF-I-immunoreactive neurones in barramundi and tilapia are sparsely scattered and their distribution exhibits pronounced inter-individual differences. In juvenile (this study) and adult tilapia (Reinecke et al. 1997) and adult daddy sculpin (Loffing-Cueni et al. 1998) brain, only IGF-I producing Purkinje cells in cerebellum are constantly present. In agreement with the potential autocrine/paracrine action of neuronal IGF-I in adult brown trout, *Salmo trutta*, IGF-I binding in the brain is highest in the cerebellum (Smith et al. 2005). The pronounced inter-individual variations of IGF-I producing neurones in adult fish brain make a neurotransmitter or -modulator function of IGF-I unlikely. Rather, neuronal IGF-I may support the survival of neurones and glia cells, as it is also likely for mammals (Cheng et al. 2001). During development, IGF-I may stimulate neurogenesis, dendritic growth and synaptogenesis in an autocrine/paracrine manner, as has been shown in mammals (e.g. Zhou et al. 1999; Niblock et al. 2000; Kakizawa et al. 2003). This hypothesis is supported by the observation that, in early developing tilapia larvae, most neurones expressed IGF-I mRNA and that the number of neurones containing IGF-I mRNA decreases with age. Whether the assumed actions of IGF-I are exerted under the control of GH is unclear at present, since GH significantly increased brain IGF-I mRNA in rainbow trout (Biga et al. 2004), whereas no effect has been reported in common carp (Vong et al. 2003).

The present study is the first to show that IGF-I occurs in the bony fish pituitary. IGF-I was observed in both the neuro- and adenopituitary. In the posterior part, neurosecretory axons containing IGF-I-immunoreactivity appear around 17 DPF, whereas no IGF-I mRNA is detected at this stage. The likely source of IGF-I in the neuropituitary is the neuronal perikarya that lie within the hypothalamus and that exhibit IGF-I immunoreactivity. In the adenopituitary, IGF-I mRNA was first detected at 20 DPF. IGF-I immunoreactivity and mRNA in endocrine cells are present throughout development. To date, IGF-I-immunoreactivity has only been localized in the anterior pituitary of the frog *Xenopus laevis*

in which it occurs in co-existence with prolactin (David et al. 2000). Because IGF-I binding sites have also been found in *X. laevis* anterior pituitary, IGF-I from the prolactin cells has been postulated to regulate GH in a paracrine manner (David et al. 2000). Whether this hypothesis also applies for bony fish remains to be clarified, as does the type of endocrine cells producing IGF-I.

At 5 DPF, IGF-I was detected in cells of the epidermis of tilapia. The number of IGF-I producing epidermal cells first increases with age reaching the highest level around 19-29 DPF. In older larvae and adults, some superficial and basal epidermal cells exhibit IGF-I immunoreactivity or IGF-I mRNA. In developing skin of shi drum, IGF-1R immunoreactivity is detectable even at hatching (Radaelli et al. 2003). The early appearance of both IGF-I and its receptor suggests an important auto/paracrine function of IGF-I in skin development. Because some IGF-I producing cells still occur in the basal and superficial layers in juvenile and adult tilapia and as high levels of IGF receptor mRNA were found in adult gilthead seabream skin (Perrot et al. 1999), local IGF-I in fish skin seems to play a role in later life. Skin IGF-I might be involved in repair mechanisms and wound healing as it has been proposed in mammals (Edmundson et al. 2003).

In summary, the expression of IGF-I is more pronounced during ontogeny than in juvenile and adult life in the parenchymal cells of most of the organs investigated, such as epithelial cells of the gastro-intestinal tract, acinar cells of the exocrine pancreas, skeletal muscle cells, cardiomyocytes, renal tubular cells, neurones of the central and peripheral nervous system and skin cells. These results suggest a high functional impact of local IGF-I in early fish growth, metabolism and organogenesis by auto/paracrine means of regulation. Whether IGF-I expression in the early development of fish is regulated by GH remains to be clarified (Perrot et al. 1999; Deane et al. 2003), although low levels of GH mRNA have been detected as early as gastrulation in rainbow trout and intermediate amounts of GH mRNA have been observed in early cleavage-stage embryos (Yang et al. 1999).

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Chapter 2

Differential expression of IGF-I mRNA and peptide in the male and female gonad during early development of a bony fish, the tilapia *Oreochromis niloticus*[*]

Insulin-like growth factor I (IGF-I) plays a key role in the complex system that regulates bony fish growth, differentiation, and reproduction. The major source of circulating IGF-I is liver, but IGF-I-producing cells also occur in other organs, including the gonads. Because no data are available on the potential production sites of IGF-I in gonad development, developmental stages of monosex breedings of male and female tilapia from 0 day post fertilization (DPF) to 90 DPF were investigated for the production sites of IGF-I at the peptide (immunohistochemistry) and mRNA (in situ hybridization) level. IGF-I mRNA first appeared in somatic cells of the male and female gonad anlage at 7 DPF followed by IGF-I peptide around 9-10 DPF. Gonad anlagen were detected from 7 DPF. Starting at 7 DPF, IGF-I peptide but no IGF-I mRNA was observed in male and female primordial germ cells (PGCs) provided that IGF-I mRNA was not under the detection level, this observation may suggest that IGF-I originates from the somatic cells and is transferred to the PGCs or is of maternal origin. While in female germ cells IGF-I mRNA and peptide appeared at 29 DPF, in male germ cells both were detected as late as at 51–53 DPF. It is assumed that the production of IGF-I in the germ cells is linked to the onset of meiosis that in tilapia ovary starts at around 28 DPF and in testes at around 52–53 DPF. In adult testis, IGF-I mRNA and peptide occurred in the majority of spermatogonia and spermatocytes as well as in Leydig cells, the latter indicating a role of IGF-I in the synthesis of male sex steroids. In adult ovary, IGF-I mRNA and IGF-I peptide were always present in small and previtellogenic oocytes but only IGF-I peptide infrequently occurred in oocytes at the later stages. IGF-I expression appeared in numerous granulosa and some theca cells of follicles at the lipid stage and persisted in follicles with mature oocytes. The results suggest a crucial role of local IGF-I in the formation, differentiation and function of tilapia gonads.

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Introduction

In fish, as in other vertebrates, the main regulators of gonadal sex differentiation appear to be steroid hormones (Nakamura et al., 1998; Piferrer, 2001). The early still undifferentiated gonad anlage of fish already possesses the capability of producing gender-specific steroids. However, sex steroids probably are not the only factors involved because recent evidence indicates that they interact with other hormonal factors in sex differentiation (Piferrer, 2001). Sexual differentiation goes along with cell proliferation and tissue growth (Nakamura et al., 1998). Thus, growth factors, especially insulin-like growth factor I (IGF-I) that exerts numerous essential functions in fish (see: Reinecke and Collet, 1998, Reinecke et al., 2005; Wood et al., 2005) may also be involved in fish gonadal development. IGF-I either may be a mediator of steroid actions or supplement the steroid effects in a quite independent manner under the control of growth hormone (GH). In this respect, the localisation of IGF-I and its receptor, the IGF-1R, in adult male, and female gonads of fish (Le Gac et al., 1996; Schmid et al., 1999; Perrot et al., 2000) indicates a functional impact of IGF-I in mature fish gonads.

Few studies have dealt with the ontogeny of IGF-I in fish. Most of them have used radioimmunological (Duan et al., 1995) or molecular biological techniques (Shamblott and Chen, 1993; Greene and Chen, 1997, 1999; Ayson et al., 2002; Deane et al., 2003). Although these investigations cannot give any information on the producing cells they suggest a functional role of IGF-I in developing fish. Furthermore, morphological studies on IGF-I using immunohistochemistry and/or in situ hybridisation (Richardson et al., 1995; Funkenstein et al., 1997; Perrot el al., 1999; Radaelli et al., 2003) have examined IGF-I ontogeny in various organs, however, to date IGF-I ontogeny in the gonads has not been investigated.

Because no information is available on the potential production sites of IGF-I in fish gonad during larval development, male and female early developmental stages of monosex tilapia (*Oreochromis niloticus*), i.e., from 0 day post fertilization (DPF) to 90 DPF, as well as adult individuals were investigated. The study uses immunohistochemistry to localize the IGF-I peptide and in situ hybridization for the presence of IGF-I mRNA.

Materials and methods

Animals

Monosex breedings of *O. niloticus* generated as described before (Baroiller et al., 1999) were used for this study. Fish larvae of both sexes at different developmental stages, i.e., from 0 to 90 days postfertilization (DPF), as well as adults were sampled and anaesthetized with 2-phenoxy-ethanol (Sigma, St. Louis, MO, USA) added to water. With the earliest stages, the trunk was cut to allow fixation solution to enter. From 70 DPF on, gonad samples were excised. Tissue preparations for in situ hybridization were fixed by immersion with 4% buffered paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and those for immunohistochemistry with Bouin's solution without acetic acid for 4 h at room temperature. Specimens were dehydrated in ascending series of ethanol and routinely embedded in paraplast (58 °C).

Generation of tilapia specific probes

Probes used for in situ hybridization were prepared as already described (Schmid et al., 1999). In brief, total RNA from tilapia liver was extracted by the phenol/chloroform method with the Ultraspec Extraction Kit (ams, Lugano, Switzerland). For cDNA synthesis, 5 μg RNA was annealed with 1 μM of a poly(dT) primer (5′-CCTGAATTCTAGAGCTCAT(dT17)-3′) for 3 min at 70 °C. The RNA/primer mix was incubated for 1 h at 37 °C with 15 mM dNTPs and 10 U AMV-reverse transcriptase (Pharmacia, Switzerland) in 1× reaction buffer (50 mM Tris–HCl, pH 8.3, 40 mM KCl, and 6 mM $MgCl_2$). One microliter of cDNA was incubated with 1 μM of the sense (5′-GTCTGTGGAGAGCGAGGCTTT-3′) and antisense primer (5′-AACCTTGGGTGCTCTTGGCATG-3′), corresponding to the B- and E-Domains, 200 μM dNTPs, and 1 U *Taq*-polymerase (Pharmacia) in 1× incubation buffer (10 mM Tris–HCl, pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatine). The amplification program was optimized for a Stratagene RoboCycler Gradient 40 as follows: one cycle of 10 min at 94 °C, 1 min at 59 °C, 2 min at 72 °C; 30 cycles of 1 min at 94 °C, 1 min at 59 °C, and 2 min at 72 °C followed by a final extension step of 5 min at 72 °C. PCR fragments were separated on a 2% agarose gel and eluted by the Gel Extraction Kit QIAquick (Qiagen). PCR products were cloned in a pCR-Script SK(+) cloning vector (Stratagene, Heidelberg, Germany).

Plasmids with the IGF-I fragment were sequenced (Microsynth, Switzerland) and the sequences compared to database. The *Escherichia coli* cells containing the plasmids were kept in glycerol stock vials. Traces of *E. coli* cells were removed with a sterile pipette tip, stroked out on 1.5% Luria–Bertani (LB)-agar plates containing ampicillin (100 μg/ml) and the plates incubated at 37 °C overnight. From each plate a single colony was picked and inoculated in 5 ml LB medium containing ampicillin. After growth for 8 h the starter culture was diluted 1:500 in LB medium and expanded with vigorous shaking overnight. The bacterial pellet was harvested by centrifugation at 6000*g* for 15 min at 4 °C.

Plasmids were purified using the Midi Purification Kit (Qiagen). The plasmids containing the specific inserts of IGF-I (207 bp), were used as templates for the synthesis of the digoxigenin (DIG)-labeled RNA probes. Linearization was performed with the restriction enzymes *Eco*RI for T3 polymerase-driven transcription and *Not*I for T7 polymerase-driven transcription. After ethanol precipitation, linearization efficiency was assured on a 1.2% agarose gel by loading 1 μg of linearized plasmid and comparison with a 1 kb DNA ladder (Promega). One microgram of the linearized plasmids was transcribed from the T3 and T7 promotors to obtain the antisense and sense probes, using the in vitro transcription kit (Roche Diagnostics, Germany) in the presence of DIG-UTP. Integrity of probes and efficiency of labelling were confirmed by gel electrophoresis, including blotting and incubation with antibody (Ab), and by dot blot. For dot blot, 1 μl of different dilutions (undiluted, 1:10, 1:100, and 1:1000) of probes and control RNAs were dropped on a nylon membrane (Roche Diagnostics) and fixed by UV cross-linking (254 nm, 125 mJ). The membrane was washed with 1% blocking reagent (Roche Diagnostics) in buffer P1 for 30 min followed by treatment with the alkaline phosphatase (AP)-coupled anti-DIG Ab (Roche Diagnostics) diluted at 1:2000 with 1% blocking reagent in P1 for 30 min. After rinsing with P1 the membrane was equilibrated with buffer P3 and finally washed with 1:50 diluted NBT/BCIP stock solution (Roche Diagnostics) until color development. For gel electrophoresis and blotting, 200 ng of the probes was loaded on a 1% denaturing formaldehyde agarose gel in 1× MOPS and run at 80 V for 1.5 h. After the run, gel and membrane were shaken in DEPC-H_2O for 5 min. Capillary transfer was carried out with 20× SSC overnight. Nucleic acids were fixed by UV crosslinking. Subsequently, the membrane was incubated with the AP-labeled anti-DIG Ab

applying the same procedure as for dot blot. Lengths of the probes were compared with control RNA (760 nt, Roche Diagnostics).

In situ hybridization protocol

Sections were cut at 4 μm, mounted on Super Frost Plus slides (Menzel-Gläser, Germany) and dried overnight at 42 °C. After dewaxing and rehydration, the sections were fixed with 4% PFA and 0.1% glutaraldehyde in 1× PBS. The following steps were carried out with DEPC-treated solutions in a humified chamber. To denaturate proteins, the sections were digested with 0.02% proteinase K in 20 mM Tris–HCl, pH 7.4, 2 mM $CaCl_2$ for 10 min at 37 °C. To reduce background, the sections were treated with 1.5% triethanolamine and 0.25% acid anhydride for 10 min at room temperature. The slides were incubated with 50 μl prehybridization solution per section for 3–4 h at 54 °C. Hybridization was carried out overnight at 54 °C with 30 μl of hybridization buffer containing 10 ng of sense or antisense probe previously denaturated for 5 min at 85 °C. Slides were washed for 15 min at room temperature in 2× SSC, and for 30 min at the specific hybridization temperature at descending concentrations of SSC (2, 1, 0.5, and 0.2×). Sections were incubated with the DIG AP-coupled Ab diluted 1:4000 in 1% blocking reagent (Roche Diagnostics) in buffer P1 for 1 h at room temperature in the dark. After washing twice in P1 for 15 min, the sections were treated with buffer P3, 5 mM levamisole and NBT/BCIP stock solution (Roche Diagnostics). The color development was carried out overnight at room temperature. The reaction was stopped by rinse of the slides in tap water for 15 min. Sections were mounted with glycergel.

Immunohistochemistry

Sections were cut at 4 μm, mounted onto glass slides (Menzel-Gläser), and dried overnight at 42 °C. After dewaxing and rehydration, they were used for immunohistochemistry. To reduce unspecific-binding sections were treated with PBS (pH 7.4) containing 2% bovine serum albumin (BSA) for 30 min at room temperature. The sections were incubated overnight with the rabbit antiserum 116 (Reinecke et al., 1997; Schmid et al., 1999) diluted at 1:400 and washed repetitively in PBS. The IGF-I antiserum was detected by incubation with biotinylated goat anti-rabbit IgG (Bioscience Products, Emmenbrücke, Switzerland, 1:100) for 30 min at room temperature. After repetitive rinse in PBS, the sections were incubated with

streptavidin–fluorescein–isothiocyanate (FITC) (Bioscience Products, 1:100) for 30 min at room temperature in the dark. Specificity of the reactions obtained was tested using the following controls: (1) Replacement of the primary antiserum by non-immune rabbit serum. (2) Pre-absorption of the primary antiserum with recombinant human (h) IGF-I, hIGF-II, porcine insulin (kind gift of Prof. J. Zapf, Zürich) or the peptide used for immunization (40, 400 μg peptide/ml diluted antiserum).

Photomicroscopy was performed with a Zeiss Axioscope using the Axiovision software 3.1. (Zeiss, Zürich, Switzerland).

Results

Specificity of detection systems

The reaction properties of IGF-I antiserum 116 diluted at 1:400 (Fig. 1A) were tested by preabsorption with IGF-I and IGF-II. While the immunoreactions were extinguished after preabsorption with 40 μg IGF-I/ml (Fig. 1B) they still were present after pre-absorption with 400 μg IGF-II/ml (Fig. 1C). Thus, the reactions obtained were specific for IGF-I.

In situ hybridization with the antisense and sense probes specific for tilapia IGF-I mRNA revealed that hybridization with the antisense probe (Fig. 1D) resulted in positive responses, whereas no signal was obtained in the negative control (Fig. 1E).

Male gonad

Gonad anlagen were first detectable at 7 DPF. At 7 DPF, IGF-I mRNA was first detected in the developing male gonad where it was present only in somatic cells but not in primordial germ cells (Fig. 2B). At 9 DPF, IGF-I immunoreactivity was also observed in somatic cells (Table 1). Starting at 7 DPF, IGF-I peptide but no IGF-I mRNA was observed in primordial germ cells (Figs. 2A and B). The exclusive occurrence of both IGF-I mRNA and peptide in somatic cells persisted until around 51 DPF, as representatively shown for 29 DPF (Figs. 2C and D). From 51 to 53 DPF on (Table 1), IGF-I mRNA (Fig. 2F) and immunoreactivity (Fig. 2E) were present in both somatic and germ cells. In adult testis, IGF-I mRNA and peptide occurred mainly in spermatogonia and spermatocytes and in Leydig cells (Figs. 2G and H).

Female gonad

Gonad anlagen were detectable from 7 DPF. In the developing female gonad, IGF-I mRNA was detected in somatic cells at 7 DPF (Table 1) followed by IGF-I peptide at 10 DPF (Fig. 3A). In contrast, only IGF-I immunoreactivity was found in primordial germ cells starting at 7 DPF (Table 1), as representatively shown for 10 and 12 DPF (Figs. 3A and C) while IGF-I mRNA was still exclusively present in somatic cells (Figs. 3B and D). At 29 DPF, IGF-I mRNA (Fig. 3F) in addition to IGF-I peptide (Fig. 3E) appeared in female germ cells. In the adult ovary (Figs. 3G–I), the expression of IGF-I varied between the different maturing follicle stages. Both IGF-I mRNA and IGF-I immunoreactivity were present in primary and previtellogenic oocytes. In oocytes of the later stages no IGF-I mRNA but occasionally IGF-I peptide (Fig. 3G) was detected in the cell periphery. Around vitellogenic oocytes and oocytes at the lipid stage, IGF-I expression was observed in numerous granulosa (Figs. 3G–I) and occasionally also in theca cells (Fig. 3H).

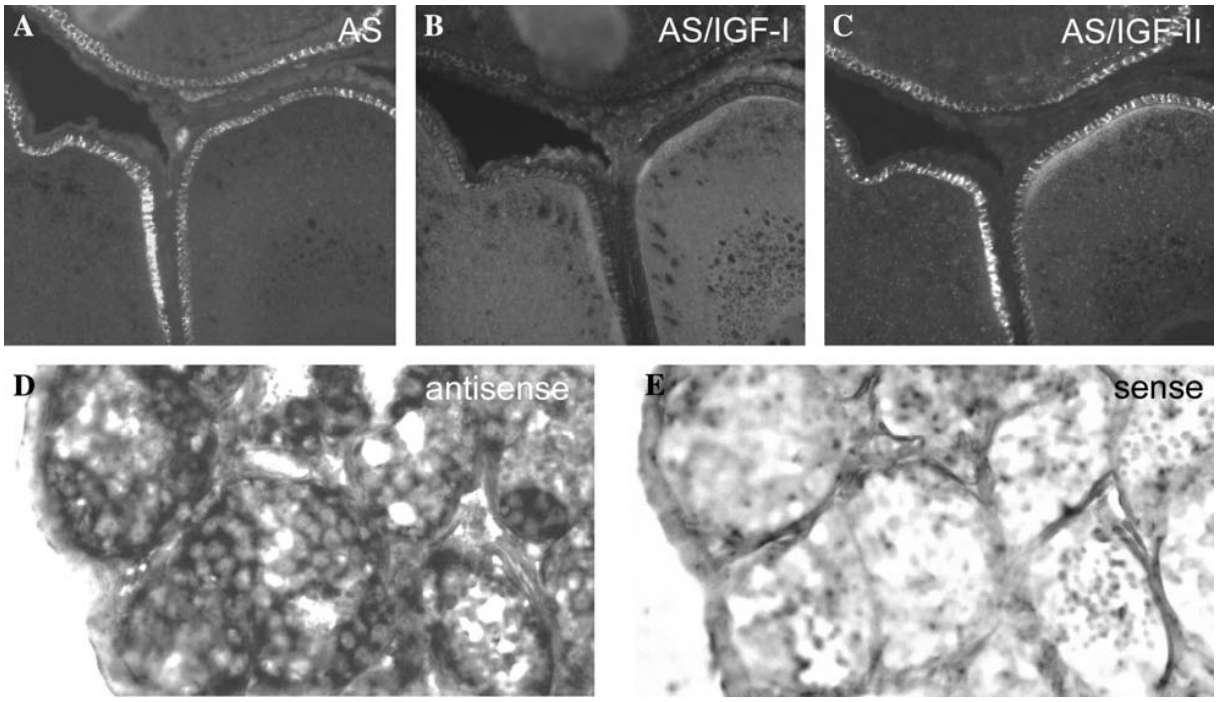

Fig. 1 Specificity of immunofluorescence (A–C) and in situ hybridization (D and E). (A–C) Pre-absorption experiment with antiserum (AS) 116 diluted at 1:400 on three consecutive sections of adult tilapia ovary. The first section (A) is incubated with AS 116, the second (B) with AS 116 preabsorbed with 40 μg IGF-I/ml, and the third (C) with 400 μg IGF-II/ml. While the immunoreactions are abolished after pre-absorption with IGF-I (B) they still are present after pre-absorption with IGF-II (C) 450×. (D and E) In situ hybridization of adult tilapia testes on two adjacent sections with an IGF-I antisense probe (D) and an IGF-I sense probe (E). Hybridization with the antisense probe specific for tilapia IGF-I mRNA shows positive responses (D), whereas no signal is present in the negative control (E). 800×.

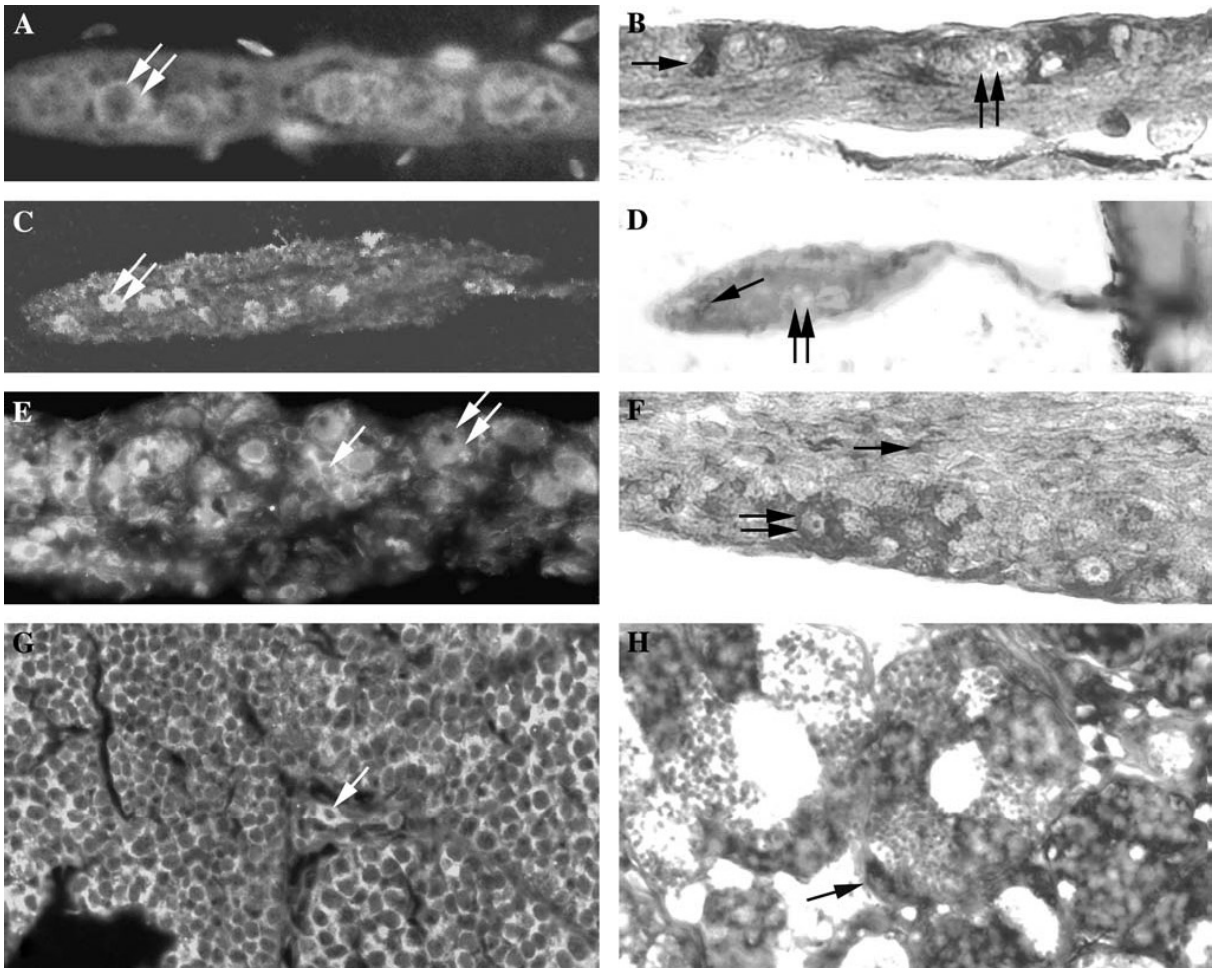

Fig. 2 IGF-I immunofluorescence (A, C, E, and G) and in situ hybridization (B, D, F, and H) in the early developing and adult male gonad. (A and B) At 7 DPF, in primordial germ cells (double arrows) only IGF-immunoreactivity (A) but no IGF-I mRNA (B) is present. IGF-I mRNA occurs in somatic cells only (arrow). (A and B) 800×. (C and D) At 29 DPF, still the primordial germ cells (double arrows) show only IGF-I immunoreactivity (C) while IGF-I mRNA is present in somatic cells (D, arrow). (C) 400×, (D) 480×. (E and F) At 52 DPF, both IGF-I peptide and mRNA occur in somatic cells (arrows) but in the spermatogonia (double arrows) in addition to IGF-I peptide (E) also IGF-I mRNA (F) is detected. (E) 500×, (F) 600×. (G and H) Adult testis. Numerous spermatogonia and spermatocytes exhibit IGF-I peptide and mRNA. Furthermore, IGF-I peptide (G) and mRNA (H) occur in Leydig cells (arrows). (G) 700×, (H) 600×.

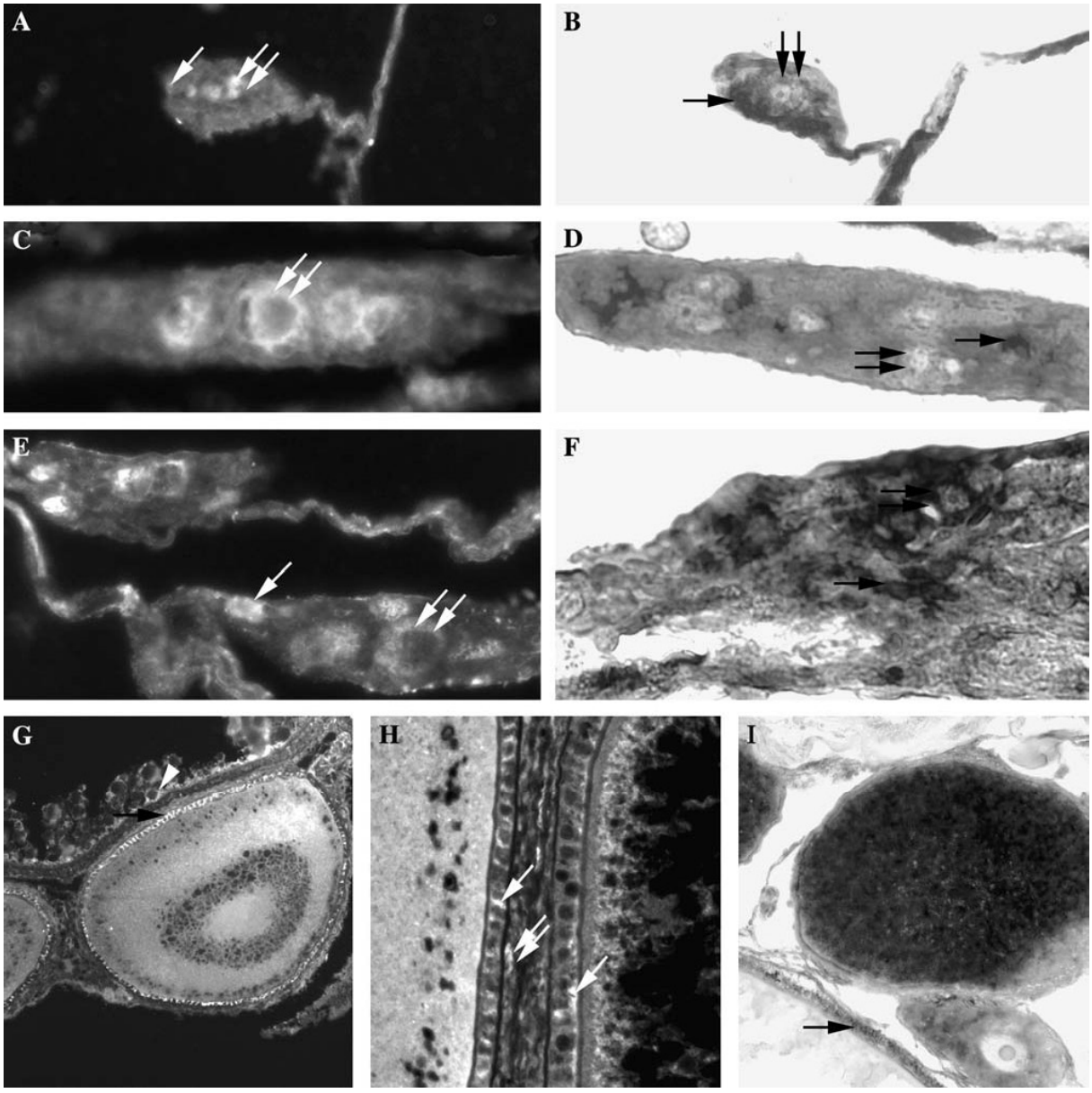
A
B
C
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Table 1
First appearance of IGF-I peptide (immunofluorescence) and IGF-I mRNA (in situ hybridization) in the developing male and female gonads

Organ	Day postfertilization IGF-I peptide	Day postfertilization IGF-I mRNA
Gonads male		
Somatic cells	9	7
Primordial germ cells	7	—
Germ cells	52–53	51–52
Gonads female		
Somatic cells	10	7
Primordial germ cells	7	—
Germ cells	29	29

Table 1 First appearance of IGF-I peptide (immunofluorescence) and IGF-I mRNA (in situ hybridization) in the developing male and female gonads

Fig. 3 IGF-I immunofluorescence (A, C, E, G, and H) and in situ hybridization (B, D, F, and I) in the early developing and adult female gonad. (A and B) At 10 DPF, in primordial germ cells (double arrows) IGF-I peptide (A) but no IGF-I mRNA (B) is present. IGF-I mRNA is found in somatic cells only (B, arrow) which also exhibit IGF-I peptide (A, arrow). (A) 500×, (B) 700×. (C and D) At 12 DPF, still in the primordial germ cells (double arrows) only IGF-I immunoreactivity (C) is present while the somatic cells also exhibit IGF-I mRNA (D, arrow). (C) 900×, (D) 700×. (E and F) At 29 DPF, both IGF-I peptide (E) and mRNA (F) occur not only in somatic cells (arrow) but also in oogonia (double arrows). (E) 800×, (F) 600×. (G–I) Adult ovary. IGF-I peptide (G and H) and mRNA (I) occur in cells of the granulosa (arrows). Furthermore, the presence of IGF-I peptide in the cell periphery of a vitellogenic oocyte (G, arrowhead) and in theca cells (H, double arrows) is shown. (G) 250× (H) 700×, (I) 400×.

Discussion

By the use of molecular biological techniques, both IGF-I (Duan et al., 1995; Duguay et al., 1996; Greene and Chen, 1997; Deane et al., 2003) and its receptor, the IGF-1R (Greene and Chen, 1999; Ayaso et al., 2002), have been detected in embryos of several fish species. These results indicate that the important components of the IGF system exist during early development and, thus, suggest a high organizational impact of IGF-I during teleost ontogeny. Our results showing the presence of IGF-I mRNA and/or peptide in the early developing gonad of tilapia strengthen this hypothesis.

Both in males and females, IGF-I mRNA and peptide appeared in somatic cells of the early gonad anlage already at 7 DPF and 9–10 DPF, respectively. Because at this stage the gonad anlage is still undifferentiated IGF-I in the somatic cells may be of crucial importance in further gonad development and differentiation. The very early expression of IGF-I in somatic cells may well be under the control of GH. In rainbow trout, GH was expressed immediately after hatching prior to the formation of the pituitary gland indicating the expression of GH in extrapituitary sites during early development (Yang et al., 1999; Deane et al., 2003) that contrasts the situation in adult fish (Caelers et al., 2005). In contrast to somatic cells, in male and female primordial germ cells only IGF-I peptide but no IGF-I mRNA was detected before 29 DPF in females and 51 DPF in males. On the one hand, IGF-I mRNA may have been under the detection level of our in situ hybridization. On the other hand, the observations may suggest that the primordial germ cells themselves do not produce IGF-I during the first weeks of development. In this case two possibilities apply to explain the presence of IGF-I: IGF-I may originate from somatic cells and be transferred to the primordial germ cells or it may be of maternal origin. In female germ cells, IGF-I mRNA and peptide were detected at 29 DPF while in male germ cells both IGF-I peptide and mRNA appeared not before 51–53 DPF. In the tilapia *O. niloticus*, the first meioses in the ovary occur around 28 DPF and in the testes around 52–53 DPF. Therefore, the appearance of IGF-I in the germ cells may be linked to the onset of meiosis.

The expression of IGF-I mRNA in isolated trout spermatogenic and Sertoli cells (Le Gac et al., 1996) is consistent with the presence of IGF-I-immunoreactivity in tilapia spermatogonia and spermatocytes as shown here and in Sertoli cells (Reinecke et al., 1997).

In agreement with the potential auto/paracrine function of IGF-I, the IGF-1R has been identified in trout testis, where it was probably located at spermatogonia and Sertoli cells (Le Gac et al., 1996). In addition, the present study shows that in fish testis IGF-I is produced in Leydig cells as it is in mammals (Lin et al., 1990) where it is thought to be a critical autocrine and/or paracrine factor in the control of adult Leydig cell numbers and function, especially in steroidogenesis (Wang and Hardy, 2004). It is reasonable to assume that IGF-I in fish testis is also involved in the synthesis of male sex steroids.

Several studies indicate further physiological roles of IGF-I in adult fish male gonad. In Japanese eel cultured testes, IGF-I stimulated all stages of spermatogenesis induced by 11-ketosterone (Nader et al., 1999). In rainbow trout, the amount of testicular IGF-I increased after GH-treatment (Le Gac et al., 1996; Biga et al., 2004) which is compatible with the presence of GH receptors in trout testis (Le Gac et al., 1996). Not only GH but also IGF-I stimulated the incorporation of thymidine into spermatogonia and primary spermatocytes from cultured spermatogenic rainbow trout testis (Loir and Le Gac, 1994; Loir, 1999) that in gilthead seabream have been shown to exhibit the IGF-1R (Perrot et al., 2000). Based on these data it can be assumed, that IGF-I serves as paracrine/autocrine regulator of fish spermatogenesis thereby interacting with steroid hormones. Furthermore, IGF-I likely mediates the action of GH on the male gonad (Biga et al., 2004).

Some in vitro studies indicate different physiological roles of IGF-I in fish ovary. IGF-I stimulated thymidine incorporation into goldfish vitellogenic follicles (Srivastava and Van der Kraak, 1994) and induced final oocyte maturation in red seabream (Kagawa et al., 1994). IGF-I increased germinal vesicle migration and breakdown, a marker for resumption of meiosis, in oocytes of ovarian fragments of striped bass, *Morone saxatilis* (Weber and Sullivan, 2000). The density of gap junctions between granulosa cells and between granulosa cell and oocytes—which both are rare in incompetent follicles—was markedly increased by IGF-I in red seabream (Patino and Kagawa, 1999). In summary, the above studies indicate that IGF-I promotes follicle cell proliferation and oocyte maturation in ovary.

IGF-I likely also plays a role in the regulation of ovarian steroidogenesis, because it inhibited steroid production (testosterone, 17α-hydroxyprogesterone) by the theca cells and stimulated steroid production (17β-estradiol, 17α, and 20β-dihydroxy-4-pregnen-3-one) by the granulosa cells in the preovulatory coho salmon ovary (Maestro et al., 1997b). Similarly,

estrogen and progesterone biosynthesis were differentially regulated by IGF-I in cultures of common carp ovarian follicles (Behl and Pandey, 1999). In agreement with the potential physiological role of IGF-I in the female gonad, the IGF-1R was localised at granulosa and theca cells of Coho salmon (Maestro et al., 1997a) and in gilthead seabream additionally at previtellogenic oocytes (Perrot et al., 2000).

The above described in vitro actions of IGF-I on oocyte proliferation and maturation as well as on steroid synthesis under physiological conditions may well be exerted by circulating (liver-derived) IGF-I, but further potentially paracrine acting candidates are the granulosa cells. These have been shown to produce IGF-I in red seabream (Kagawa et al., 1995), gilthead seabream (Perrot et al., 2000) and tilapia (Schmid et al., 1999, this study). Whether these actions are under the control of GH at present is unclear, however, an increase of IGF-I in the gonad after GH application has been shown in rainbow trout (Biga et al., 2004)

IGF-I mRNA was further present in young and previtellogenic oocytes as shown here but did not occur in oocytes at later stages of development (Schmid et al., 1999; Perrot et al., 2000, this study). Thus, the intraovarian IGF-I production seems to switch from the young oocyte to the surrounding follicle cells during development (Schmid et al., 1999). Occasionally, in the present study IGF-I peptide was also observed in mature oocytes. This may have been delivered by the surrounding granulosa cells. However, another possibility also applies. In unfertilized eggs of seabream a weak expression of IGF-I mRNA has been reported (Greene and Chen, 1997, Perrot et al., 1999) while no IGF-I mRNA was detected in unfertilized eggs of rabbitfish (Ayson et al., 2002). Thus, mature oocytes may transiently express IGF-I and store the hormone for later use.

In the Japanese quail, IGF-I has been shown to act not only as endocrine but also as paracrine/autocrine regulator of gonadal growth and differentiation (Fu et al., 2001) while experimental data on a role of IGF-I in gonad differentiation in bony fish are lacking to date. However, the present study by showing distinct and time-dependent IGF-I production sites in male and female tilapia gonads during ontogeny suggests a similar role for IGF-I also in fish gonads.

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Indication for a Particular Physiological Impact of IGF-I and its Organ-specificity During Early Development of Bony Fish Revealed in the Tilapia, *Oreochromis niloticus*, by In situ Hybridisation and Immunohistochemistry. Seminar über aktuelle Probleme der morphologischen Forschung (Vorlesung 526), Institute of Anatomy, University of Zürich, 21.12.2005.

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